Patent
176668.1

13017-3

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Before the Board of Patent Appeals and Interferences

In re Appeal regarding Patent Application of

Applicants : GOELET, Philip *et al.*

Examiner: MYERS, Carla J.

Application No.: 09/258,132

Art Unit: 1634

Filing Date: 26 February 1999

Title: Nucleic Acid Typing By Polymerase Extension of Oligonucleotides Using Terminator Mixtures

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APPEAL BRIEF

I. <u>Real Party in Interest</u>

The real party in interest with respect to the application on appeal; application No. 09/258,132; is the assignee of record: Orchid Cellmark Inc.; a corporation of Delaware having a place of business at 4390 US Route One, Princeton, NJ 08540.

II. Related Appeals and Interferences

The subject application on appeal is a continuation of application No. 07/664,837, filed 5 March 1991 ("the '837 application"), now patent 5,888,819 ("the '819 patent").

The subject application for which the present appeal is pending on appeal has not been the subject of an interference or any judicial proceeding.

The '837 application was involved in two interferences: interference No. 103,562 and interference No. 103,739.

Application 07/775,786, filed 11 October 1991 ("the '786 application"), now patent 6,004,744 ("the '744 patent"), was a continuation-in-part of the '837 application.

Application 09/258,133, filed 26 February 1999, now patent 6,537,748 ("the '748 patent"), was continuation of the '786 application and a continuation-in-part of the '837 application.

The '819 patent, the '744 patent, and the '748 patent are the subject of a pending patent infringement lawsuit: Beckman Coulter Inc. and Orchid Cellmark Inc. v. Sequenom, Inc., No. 08 CV 1013 W POR (S.D. Cal. 5 June 2008).

III. Status of Claims

Claims 1 – 63 (Cancelled without prejudice)

Claim 64 (Finally rejected; on appeal)

Claim 65 (Cancelled without prejudice)

Claims 66 – 71 (Finally rejected; on appeal)

IV. Status of Amendments

No amendment to the claims or specification to the subject application has been filed subsequent to the final Office Action of 22 May 2007 on appeal.

The following papers have been filed in connection with the application subsequent to the 22 May 2007 Office Action on appeal: a notice of appeal submitted by mail with a certificate of mailing dated 21 November 2007 and a petition for an extension of time under 37 CFR 1.136(a) to respond to the Office Action through 22 November 2007, also submitted by mail with a certificate of mailing dated 21 November 2007.

V. Summary of Claimed Subject Matter

The subject application has a single independent claim: claim 64; which has been finally rejected in the Office Action of 22 May 2007 on appeal. Claim 64 is summarized below, with references to the page and line numbers of the specification of the application as filed and to the drawings as filed by reference characters, in accordance with the requirements of 37 CFR 41.37(v). References to the specification of the application below are in the format [*page No. 1:line No. 1 – line No. 2*] or [*page No.1:line No. 1 – page No. 2:line No. 2*].

Independent claim 64 is directed to a method of determining the identity of one or more nucleotide bases at a plurality of specific positions in one or more nucleic acid molecules of interest [11:11-17, 25-32; 11:34-12:9; 27:26-34; 28:27-30].

The method of claim 64 comprises the step of treating a sample comprising the nucleic acid molecules of interest if the nucleic acid molecules of interest are double-stranded so as to obtain unpaired nucleotide bases spanning the specific positions. Alternatively, a sample of the nucleic acid molecules of interest may be used directly if the nucleic acid molecules are single stranded [9:35-10:3; 11:11-17; 27:26-32].

The method of claim 64 includes the further step of contacting the sample with a plurality of different oligonucleotide primers [11:17-19; 18:20-26; 27:32-34]. Each different oligonucleotide primer hybridizes to a corresponding different stretch of nucleotide bases present in the nucleic acid molecules of interest which is immediately adjacent to the specific position of a nucleotide base to be identified with that oligonucleotide primer, so as to form a duplex such that the nucleotide base to be identified is the first unpaired base of the nucleic acid molecule of interest immediately downstream of the 3' end of the primer [10:3-14; 11:17-23; 27:2-10]. Each different oligonucleotide primer comprises a corresponding different affinity moiety [29:19-27; 29:36-30:3; 31:23-35]. The oligonucleotide primer comprising the affinity moiety is capable of hybridizing with a nucleic-acid template and undergoing a nucleic acid template-dependent primer extension reaction with terminators of a terminator reagent. The affinity moiety permits

affinity separation of the extended oligonucleotide primer from the terminator reagent [27:2-6, 12-15; 27:26-28:2; 29:19-24; 31:23-30].

The method of claim 64 also includes the step of contacting the duplexes with a terminator reagent which includes four different terminators of a nucleic acid template-dependent primer extension reaction. The terminator reagent is free of dATP, dCTP, dGTP, and dTTP. Each terminator comprises a different detectable label corresponding to the terminator [10:20-23; 11:11-19; 19:28-20:2; 21:22-28; 31:6-15, 32-35]. One of the terminators is complementary to a nucleotide base to be identified by each of the oligonucleotide primers. The contacting is carried out in a primer-extension reaction medium under conditions sufficient to permit a template dependent primer extension reaction which incorporates the complementary terminator onto the 3' end of each of the different oligonucleotide primers to thereby extend the 3' end of each of the oligonucleotide primers by one terminator [10:23-31; 11:11-23; 19:31-20:2; 27:26-34].

The method of claim 64 further includes the step of affinity separating the respective extended oligonucleotide primers from primer-extension reaction medium by causing each of the extended oligonucleotide primers to contact an affinity group attached to a solid support. The affinity group is complementary to the affinity moiety incorporated in the oligonucleotide primer [18:20-26; 27:12-15, 19-24; 29:19-27; 29:36-30:3; 31:23-35].

Finally, the method of independent claim 64 includes the step of determining the presence and identity of the nucleotide base at each of the respective specific positions in the one or more nucleic acid molecules of interest by detecting the detectable label of the terminator incorporated at the 3' end of each of the affinity separated extended oligonucleotide primers [10:31-11:2; 11:11-23; 20:2-10; 27:26-28:2; 31:23-35].

VI. Grounds of Rejection to be Reviewed on Appeal

VI.*a*) Whether claims 64, 66, 67, 69, and 70 were unpatentable under 35 U.S.C. §103(a) over European published patent application EP 0 412 883 A1 to Cohen *et al.* ("the Cohen *et al.* '883 published European application") or French patent 2,650,840 also to Cohen *et al.* ("the Cohen *et al.* '840 French patent"), each in view of international PCT published patent application WO 90/11372 to Davis *et al.* ("the Davis *et al.* '372 PCT published application"), particularly if proper account were taken, as required by United States Supreme Court precedent, of teachings in the Cohen *et al.* patent documents against the hypothetical combination proposed in the Office Action on appeal of the mobile-phase analytical method disclosed in the Cohen *et al.* documents with the analytical method involving immobilization on a membrane substrate disclosed in the Davis *et al.* '372 PCT published application.

VI.*b*) Whether claim 68 was unpatentable under 35 U.S.C. § 103(a) over the Cohen *et al.* '883 published European application or the Cohen *et al.* '840 French patent, each in view of the Davis *et al.* '372 PCT published application and United States patent No. 5,332,666 to Prober *et al.* ("the Prober *et al.* '666 patent"), particularly if proper account were taken, as required by United States Supreme Court precedent, of teachings in the Cohen *et al.* patent documents against the hypothetical combination proposed in the Office Action on appeal of the mobile-phase analytical method disclosed in the Cohen *et al.* documents with the analytical method involving immobilization on a membrane substrate disclosed in the Davis *et al.* '372 PCT published application.

VI.*c*) Whether claim 71 was unpatentable under 35 U.S.C. § 103(a) over the Cohen *et al.* '883 published European application or the Cohen *et al.* '840 French patent, each in view of the Davis *et al.* '372 PCT published application and United States patent No. 4,962,020 to Tabor *et al.* ("the Tabor *et al.* '020 patent"), particularly if proper account were taken, as required by United States Supreme Court precedent, of teachings in the Cohen *et al.* patent documents against the hypothetical combination proposed in the Office Action on appeal of the mobile-phase analytical method disclosed in the Cohen *et al.* documents with the analytical method

involving immobilization on a membrane substrate disclosed in the Davis *et al.* '372 PCT published application.

VII. Argument

VII.*a.*) Preliminaries: Language of the Cohen *et al.* Patent Documents and Relationships Among the Rejected Claims

Claims 64 and 66 through 71 inclusive were finally rejected in the Office Action of 22 May 2007 under 35 U.S.C. § 103(a) as unpatentable over European published patent application EP 0 412 883 A1 to Cohen *et al.* ("the Cohen *et al.* '883 published European application") or French patent 2,650,840 also to Cohen *et al.* ("the Cohen *et al.* '840 French patent"), each in view of international PCT published patent application WO 90/11372 to Davis *et al.* ("the Davis *et al.* '372 PCT published application"), and, in the case of claim 68, further in view of the Prober *et al.* '666 patent, or, in the case of claim 71, further in view of the Tabor *et al.* '020 patent.

It was noted in the Office Action that the Cohen *et al.* '883 published European application claimed priority to a French patent application 8910802, which issued as the '840 French patent. Since both the Cohen *et al.* '883 published European application and the Cohen *et al.* '840 French patent are in the French language and since an English translation of the '840 patent has been provided in the present case, only the '840 French patent as translated will be referred to specifically in the remarks which follow. The undersigned attorney is not aware of any reason why the '840 French patent as translated might not accurately represent the disclosures of the French-language Cohen *et al.* '883 published European application and the French-language Cohen *et al.* '840 French patent.

Claim 64 is the sole independent claim involved in the present appeal. The remaining claims involved in the appeal: claims 66 through 71 inclusive; are dependent claims which depend upon independent claim 64 directly or indirectly.

VII.*b.*) The Cohen *et al.* '840 French Patent in View of the Davis *et al.* '372 PCT Published Application

The attorneys for the applicants contend that the Cohen *et al.* '840 French patent taught directly away from the combination, proposed in the final Office Action rejections under 35 U.S.C. § 103(a) on appeal, of the process for identifying a single base in a nucleic acid sequence of the Cohen *et al.* patent with the method of the Davis *et al.* '372 PCT published application for testing a single sample of DNA simultaneously for multiple alleles or for testing simultaneously at multiple loci for a single allele or multiple alleles.

In this regard, we point out that the Cohen *et al.* '840 French patent expressly distinguished the method of the patent from three previously known techniques for identifying a mutation in nucleic acid involving a single nucleotide position: (1) a long-probe technique, (2) a short-probe technique, and (3) the method of United States patent 4,656,127 to Mundy ("the Mundy '127 patent"). The three previously known techniques distinguished from the method of the Cohen *et al.* patent in the patent specification differed fundamentally one from another in the manner in which the single nucleotide mutation was identified using labeled oligonucleotide probes, but had in common, in embodiments to which relevant distinguishing comments in the Cohen *et al.* patent applied, steps involving detection of the labeled probes hybridized along at least a portion of their length to nucleic acid immobilized on a membrane. As discussed in detail below, in distinguishing the three previously known techniques, the Cohen *et al.* French patent taught that each technique was disadvantageous relative to the mobile-phase analysis method of the patent at least in part because, as ordinarily practiced, each of the previously known techniques involved immobilization of nucleic acid on a membrane. The method of the Davis *et al.* '372 PCT published application for testing a single sample of DNA simultaneously for multiple alleles or for testing simultaneously at multiple loci for a single allele or multiple alleles which was proposed in the Office Action on appeal as obvious to combine with the analysis method of the Cohen *et al.* '840 French patent, like the previously known techniques distinguished in the Cohen *et al.* patent, involved immobilization of nucleic acid on a membrane. It is submitted, therefore, that the hypothetical combination proposed in the Office Action on

appeal would have been understood by persons of ordinary skill in the art as of the effective filing date of the subject application as running directly counter to the teachings of the Cohen *et al.* patent. In *KSR International* v. *Teleflex* No. 04-1350, slip op. 12 (U.S. April 30, 2007), the United States Supreme Court has affirmed the "principle that when the prior art teaches away from combining certain known elements, discovery of a successful means of combining them is more likely to be nonobvious."

VII.*b*.1.) Summary of the Office Action on Appeal with Respect to the Final Rejection Under 35 U.S.C. § 103(a) of Claims 64, 66, 67, 69, and 70 as Unpatentable over the Cohen *et al.* '840 French Patent in View of the Davis *et al.* '372 PCT Published Application

It was asserted in the Office Action of 22 May 2007 on appeal that the '840 French patent disclosed a method of determining the identity of one or more nucleotide bases in a nucleic acid molecule which involved contacting a single-stranded nucleic acid sample with an oligonucleotide primer to form a duplex between the primer and complementary target nucleic acids present in the sample, wherein the primer hybridized immediately 3' of the nucleotide to be determined. It was asserted that the method of the '840 French patent further included the step of contacting the duplexes with a solution containing four different terminators, each labeled with a different detectable moiety. The method of the '840 French patent assertedly further included the steps of extending the primer with the terminator and determining the identity of the incorporated terminator to determine the identity of the nucleotide base. It was conceded in the Office Action of 22 May 2007 that the Cohen *et al.* '840 French patent did not disclose performing the primer extension reaction using multiple primers, each comprising a different affinity moiety.

It was asserted in the Office Action on appeal that the Davis *et al.* '372 PCT published application disclosed a method for determining the identity of one or more nucleotide bases in a nucleic acid molecule which comprised contacting a single-stranded nucleic acid molecule with an oligonucleotide primer to form a duplex between the primer and complementary target

nucleic acids. It was asserted that the duplexes were contacted with a solution containing labeled dNTPs to extend the primer with the dNTPs, assertedly such that if the primer were perfectly complementary with the target nucleic acid, an extension product would be formed, but if the primer contained a mismatch at or near the 3' end of the primer, an extension product would not be formed. It was asserted in the Office Action on appeal that, in the method of the '372 PCT published application, the presence of an extension product was detected in order to determine the identity of a nucleotide base. In the Office Action, it was asserted that the '372 PCT published application disclosed that the identity of multiple nucleotides could be determined simultaneously by using a mixture of different oligonucleotides, in which each oligonucleotide comprised a unique tail. It was asserted that, following the extension reaction, the primer extension/target nucleic acid complex was denatured and the primer extension product was hybridized to a solid support having bound thereto sequences complementary to the primer tail. It was asserted in the Office Action of 22 May 2007 that the unique tail allowed for the primers to be immobilized at specific locations on the support.

It was asserted in the Office Action of 22 May 2007 that it would have been obvious to have modified the method of the Cohen *et al.* '840 French patent so as to have used multiple primers, each having a different tail, and to have separated the primer extension products from the reaction medium by contacting the extension products with a solid support having immobilized thereon nucleic acid with a sequence complementary to the tail sequence – referred to in the Office Action as a "capture probe" – assertedly in order to accomplish objectives assertedly set forth in the Davis *et al.* '372 PCT published application.

VII.*b*.2.) The Cohen *et al.* '840 French Patent Taught Away from the Proposed Hypothetical Combination of the Method of the Cohen *et al.* Patent and the Method of the Davis *et al.* '372 PCT Published Application

As disclosed at page 4, line 29 through page 5, line 19 of the Cohen *et al.* '840 French patent, the process of the patent for detecting a specific nucleotide base present on a nucleic acid sequence involved hybridizing the sequence in which the base to be identified was located with a

"trigger" nucleotide which hybridized with its 3' end adjacent to the specific nucleotide base to be detected. Synthesis of the complementary strand of the resulting hybrid was initiated in the presence of a polymerase without 3'-to-5' exonuclease action and at least one modified nucleotide base capable of being incorporated into the extension product of the trigger nucleotide and of blocking further elongation of the extension product. The process of the Cohen *et al.* '840 French patent further involved detecting the incorporated blocking nucleotide base to identify the specific complementary nucleotide base located in the target nucleic acid sequence. According to page 5, lines 23 through 31 of the Cohen *et al.* patent, the blocking nucleotide bases could be dideoxynucleotides marked with radioactive substances, enzymes, fluorescent or chemoluminescent chromophoric chemical products, or antibodies.

At page 6, lines 29 through 33 of the Cohen *et al.* '840 French patent, it was disclosed that a purported advantage of the process of the patent was that the process did not require immobilization of the nucleic acid on a membrane. As may be seen, for example, at page 1, lines 5 through 13, and page 2, lines 8 through 18 of the Cohen *et al.* patent, in the context of the patent, the term "nucleic acid" applied generally to each strand of hybridized DNA or RNA, including probes 150 nucleotides long and shorter probes. Moreover, as noted in the preceding section, the necessity to immobilize nucleic acid on a membrane was specifically pointed out in the Cohen *et al.* '840 French patent to be a disadvantage shared in common by the previously-known long-probe technique and the short-probe technique – both of which techniques, as discussed below, as usually practiced involved Southern blot transfers of DNA strands from an electrophoresis gel to a membrane where the strands were immobilized – and the substantially different method of the Mundy '127 patent. See page 3, lines 10 through 17 and page 4, lines 14 through 17 of the Cohen *et al.* patent. It is submitted therefore that the Cohen *et al.* '840 French patent would have directly led persons skilled in the art away from <u>any technique</u> which shared the requirement of immobilization of nucleic acid on a membrane.

Persons of ordinary skill in the art would have recognized, it is submitted, that the multiple-allele/multiple-loci method of the Davis *et al.* '372 PCT published application was just

such a technique involving immobilization of nucleic acid on a membrane from which the Cohen *et al.* patent taught away. The Davis *et al.* published application disclosed a technique for determining the existence or nonexistence of a test nucleotide on a strand of DNA which employed a polymerization agent capable of synthesizing an extension product if there were a match between the test nucleotide on the DNA strand and a nucleotide opposite on an extension primer, but not if there were a mismatch. According to page 5, line 19 through page 6, line 22 of the Davis *et al.* '372 PCT published application, a single sample of DNA could be tested simultaneously for multiple alleles at a single locus or for a single allele or multiple alleles at multiple loci by treating the DNA with a plurality of different oligonucleotide primers, each primer being complementary to a different allele and each having a unique oligonucleotide "tail." The primers and the DNA were then subjected to conditions that would have allowed the primers and DNA to pair and labeled extension products to form if there were a match between a test nucleotide and the opposite nucleotide on the primer, but not if there were a mismatch. It was disclosed at page 6, lines 7 through 22 and page 21, lines 8 through 12 of the Davis *et al.* published application that the presence or absence of a particular extension product could be determined by applying the putative extension products to a substrate such as filter paper, nylon, or nitrocellulose "spotted" at distinct locations with unique oligonucleotides complementary to each of the unique oligonucleotide tails. As discussed in more detail below, the Davis *et al.* '372 PCT published application at page 8, lines 1 through 5, for example, disclosed that such spotting of oligonucleotides complementary to each of the unique oligonucleotide tails on the substrate preferably resulted in the oligonucleotides "being firmly bound to the substrate but accessible for hybridization with complementary sequences." According to the Davis *et al.* published application, if a particular extension product existed, it would have attached to the substrate at only one location by way of hybridization of the unique tail to the complementary oligonucleotide found only at that location on the substrate. The Davis *et al.* '372 PCT published application disclosed that by detecting the presence of a labeled extension product hybridized to a complementary oligonucleotide bound to the substrate at a specific location, the presence or absence of a specific allele in the test DNA could be determined.

The Cohen *et al.* '840 French patent drew no distinction between reversibly immobilizing nucleic acid on a membrane and irreversibly immobilizing nucleic acid on a membrane, but declared without qualification on page 6, lines 29 through 33 of the specification that an advantage of the process of the patent was that the process did not require immobilization of the nucleic acid on a membrane. Significantly in this regard, each of the three previously known techniques for identifying a single nucleotide mutation distinguished in the Cohen *et al.* patent from the process of the patent in terms of having the disadvantage of requiring nucleic acid immobilized on a membrane would, it is submitted, have been expected by persons of ordinary skill in the art generally to have involved detecting a <u>hybrid nucleic-acid complex</u> immobilized on a membrane, in which a first component of the hybrid complex was a nucleic acid effectively irreversibly bound to the membrane and a second component of the hybrid complex was a labeled oligonucleotide of some sort reversibly bound to the first component by hybridization. Moreover, as discussed below, persons of ordinary skill in the art would have recognized, it is submitted, that the method of the Davis *et al.* '372 PCT published application likewise involved detecting a hybrid nucleic-acid complex immobilized on a membrane in which a first component of the hybrid complex was a nucleic acid effectively irreversibly bound to the membrane and a second component of the hybrid complex was a labeled oligonucleotide reversibly bound to the first component by hybridization and therefore such persons would have appreciated that the method of the Davis *et al.* published application necessarily entailed a feature which the Cohen *et al.* '840 French patent characterized specifically as a disadvantage not shared by the process disclosed in the Cohen *et al.* patent. Persons of ordinary skill in the art would thus have deemed it in no way obvious to combine the process of the Cohen *et al.* patent with the method of the Davis *et al.* '372 PCT published application as proposed in the Office Action on appeal.

Turning now to the long-probe technique distinguished from the mobile-phase analysis method of the Cohen *et al.* '840 French patent in the specification of the '840 patent, persons of ordinary skill in the art would have understood the long-probe technique described in the '840 patent to be a conventional restriction-site analysis method of which the method described in E. M. Southern, *Journal of Molecular Biology*, volume 98, pages 503 through 517 (1975) ("the

Southern publication") was illustrative. The Southern publication has been made of record in the subject application and a copy of the publication is included in the evidence appendix accompanying the present brief. In the long-probe technique as applied to identifying a single nucleotide polymorphism in DNA, the polymorphism was identified by whether or not a particular restriction enzyme cut the DNA at the site of the polymorphism. Application of the restriction enzyme to the DNA was carried out in solution prior to size separation of the enzyme-treated DNA by electrophoresis, immobilization of the size-separated enzyme-treated DNA in single-strand form on a membrane, and hybridization of a labeled nucleic-acid "long probe" to the immobilized strands of the DNA to identify the physical positions on the membrane, and hence the size, of any DNA strands complementary to the probe. Reference to pages 504 through 508 of the Southern publication, and to page 506, lines 7 and 8 in particular, will show that the technique involved blot transfer of denatured DNA fragments from an electrophoresis gel to a cellulose nitrate strip, after which transfer the cellulose nitrate strip was baked in a vacuum oven for two hours at 80° C. The strip bearing the denatured DNA fragments was then treated with a solution of radioactive RNA to permit the RNA to hybridize to any complementary DNA. The strip was then washed, dried, and laid on X-ray film to detect the location of any hybrid RNA/DNA complex on the strip. Any radioactive spots on the cellulose nitrate strip detected by the X-ray film would have corresponded to a hybrid nucleic-acid complex immobilized on the strip, including, as one component, denatured DNA bound to the strip by the vacuum baking step and, as the other component, radioactively labeled RNA hybridized to the bound denatured DNA.

Transfer of size-separated DNA from an electrophoresis gel to a membrane was carried out by a "blotting" procedure described in the Southern publication, which transfer procedure is often referred to as the "Southern blot" procedure. The expression "Southern blot" procedure is also sometimes used in a second sense to refer to the entire restriction-site analysis procedure from application of the restriction enzyme to DNA in solution through determination of the positions of labeled probes hybridized to strands of blot-transferred DNA immobilized on a

membrane. The specification of the Cohen *et al.* '840 French patent appears to have used the expression "Southern blot" in both senses.

In view of the Southern publication, it is submitted that a person of ordinary skill in the art would have understood the long-probe technique described generally in the Cohen *et al.* '840 French patent at page 2, lines 24 through 34 and referred to as "the Southern blot technique" to have involved detecting a hybrid nucleic-acid complex immobilized on a membrane, in which a first component of the hybrid complex would have been a nucleic acid effectively irreversibly bound to the membrane by vacuum baking or a process of similar effect and a second component of the hybrid complex would have been a radioactively labeled oligonucleotide reversibly bound to the first component by hybridization.

The textbook *Biochemistry*, third edition, by L. Stryer at page 169 ("the Stryer textbook") provided an example of using the long-probe technique of Southern to analyze DNA for the presence of a single-nucleotide polymorphism; specifically, the sickle-cell gene. Page 169 of the Stryer textbook has been made of record in the subject application and is included in the evidence appendix accompanying the present brief. In the Office Action of 22 May 2007, it was attempted to limit the teachings of the Cohen *et al.* '840 French patent away from immobilization of nucleic acid on a membrane to cases in which hybridization of a probe to immobilized nucleic acid was used to directly detect a single nucleotide mutation – see the page 6, line 17, through page 7, line 5 of the 22 May 2007 Office Action, for example. However, the technique for analyzing for the presence of the sickle-cell gene described on page 169 of the Stryer textbook entailed the step of detecting the single-nucleotide polymorphism by digestion of DNA with a restriction enzyme, which persons of ordinary skill in the art would have recognized was to be carried out on DNA in solution, not on DNA immobilized on a membrane. According to lines 12 through 15 of page 169 of the Stryer textbook, following the digestion by the restriction enzyme, "[t]he fragments in the digested sample of DNA are separated by gel electrophoresis and visualized by Southern blotting with a ^{32}P-labeled probe that is complementary to [a] 1.1kb fragment" present in both the normal and the sickle-cell gene. [Citation omitted.] Thus, as

persons of ordinary skill in the art would have appreciated, the labeled probe used to visualize the electrophoresis pattern from the digested DNA in the gene-analysis technique described on page 169 of the Stryer textbook did not itself serve to discriminate between the two single-nucleotide polymorphisms detected by the technique, but merely marked the location of size-separated fragments on a membrane to which fragments such probes were hybridized according to the Southern blot technique.

The Cohen *et al.* '840 French patent specifically described a technique involving "long probes" in terms which persons of ordinary skill in the art would have recognized applied to the gene-analysis technique described on page 169 of the Stryer textbook discussed above. The Cohen *et al.* French patent also described a short-probe technique which, it is submitted, persons of ordinary skill in the art would have recognized to be a technique significantly different from the long-probe technique in many respects.

Turning next to a technique described generally passim from page 2, line 19 to page 3, line 17 of the Cohen *et al.* '840 French patent which involved use of a short probe in contrast to the longer probe of the long-probe technique discussed above, it is submitted that persons of ordinary skill in the art would have understood the described short-probe Southern blot technique to be an allele-specific hybridization method, of which one of two alternative allele-specific-hybridization methods described in B. J. Conner *et al.*, *Proceedings of the National Academy of Sciences USA*, vol. 80, pages 278 through 283 (January 1983) ("the Conner *et al.* publication") would have been illustrative. The Conner *et al.* publication has been made of record in the subject application and a copy of the publication is included in the evidence appendix accompanying the present brief. (The Conner *et al.* publication disclosed an alternative allele-specific hybridization procedure on page 279, right-hand-side column, lines 1 through 8, which evidently did not involve immobilization of nucleic acid on a membrane and which therefore appears to be irrelevant to the present discussion.)

According to the abstract on page 278 of the Conner *et al.* publication, two oligonucleotides nineteen bases long, one complementary to the normal human β-globin gene

and the other complementary to the sickle cell β-globin gene, could be radioactively labeled and used as probes in DNA hybridization under particular hybridization conditions. Specifically, hybridization conditions reportedly could be found such that the probes could be used to distinguish the normal gene from the sickle-cell gene on the basis of to which gene the respective probes hybridized. One method of detecting whether such allele-specific hybridization did or did not occur disclosed in the Conner *et al.* publication involved digesting a sample of DNA with a particular restriction enzyme, separating the restriction fragments on an electrophoresis gel, denaturing the DNA fragments on the gel, and transferring the denatured fragments to nitrocellulose paper "by the standard Southern procedure." See page 280, left-hand-side column, lines 2 through 7 of the Conner *et al.* publication. The reference to the standard Southern procedure quoted above was accompanied by a citation to the Southern publication discussed in the preceding paragraph. As noted in the preceding paragraph, the technique of blot transfer of denatured DNA fragments from an electrophoresis gel to a strip of nitrocellulose disclosed in the Southern publication involved baking the strip to which the fragments had been transferred in a vacuum oven.

According to page 279, left-hand-side column, line 33 through page 279, right-hand-side column, line 1 and page 279, right-hand-side column, lines 8 through 14, of the Conner *et al.* publication, hybridization of the denatured DNA fragments with the radioactively labeled oligonucleotide probes could be carried out directly on the nitrocellulose paper to which the fragments had been transferred according to the procedure of the Southern publication. Whether or not hybridization between a labeled probe and a DNA fragment on the nitrocellulose paper occurred could be detected by autoradiography after washing the paper under particular specified conditions. Any radioactive spots on the nitrocellulose paper detected by autoradiography would have corresponded to a hybrid nucleic-acid complex immobilized on the paper, including, as one component, denatured DNA bound to the paper by the vacuum baking step called for in the Southern paper referenced in the Conner *et al.* publication and, as the other component, radioactively labeled oligonucleotide probe material hybridized to the bound denatured DNA. It is submitted that a person of ordinary skill in the art would have understood the short-probe

Southern blot technique described generally in the Cohen *et al.* '840 French patent likewise to have involved detecting a hybrid nucleic-acid complex immobilized on a membrane, in which a first component of the hybrid complex would have been a nucleic acid effectively irreversibly bound to the membrane by vacuum baking or a process of similar effect and a second component of the hybrid complex would have been a radioactively labeled oligonucleotide reversibly bound to the first component by hybridization.

The disclosure of the Cohen *et al.* '840 French patent at page 2, line 19 through page 3, line 17 concerning the long-probe and short-probe techniques and certain disadvantages of the two techniques is set out below:

> Thus, to detect a mutation involving a single base, depending on the case generally two types of probes can be used: nucleic acid probes called long probes, generally over 150 nucleotides, or nucleic acid probes called short probes, generally between 17 and 24 nucleotides. If the mutation occurs at a site recognized specifically by an enzyme called a restriction enzyme, the Southern blot technique can be used. This technique includes stages of isolating the DNA, digestion by the restriction enzyme, electrophoresis on gel, transfer onto a membrane, and hybridization by means of a long probe involving the region of the mutation; after washing and autoradiography, analysis of the size of the fragments obtained permits confirmation or invalidation of the presence of the mutation. This very cumbersome process requires that the mutation involve a restriction site. If this is not the case, a short nucleotide probe of 17 to 24 nucleotides can be synthesized, the center of which coincides with the mutation that one wishes to detect. By selecting suitable hybridization and rinsing conditions (specific for each system), hybridization by means of marked oligonucleotides can be achieved only in case of perfect equivalence (the difference of a single nucleotide, particularly at the site of the mutation, results in destabilization of the hybridization).
>
> However, these various methods all have a certain number of disadvantages:
>
> – the temperature conditions are difficult to master to achieve suitable hybridization;
>
> – the mandatory presence of a restriction site may be required;
>
> – the nucleic acid is immobilized on a membrane (Southern blot).

In the preceding quotation concerning the long-probe and the short-probe methods for detecting a mutation involving a single base and disadvantages of such methods, the Cohen *et al.* '840 French patent identified three separate disadvantages and introduced the three disadvantages with the following language: "these various methods all have a certain number of disadvantages." [Underlining added.] The first and third disadvantages were stated unconditionally in the Cohen *et al.* French patent; the second disadvantage was stated as a possibility; i.e. "a restriction site *may* be required." [Emphasis added.]

In the first paragraph of the quotation from the Cohen *et al.* '840 French patent set out in the preceding paragraph, it was noted that the process involving hybridization by means of a long probe required that the mutation involve a restriction site, and that, if that were not the case, a process involving hybridization by means of a short probe could be used. In view of the first paragraph of the quotation, the second disadvantage quoted above stating that "the mandatory presence of a restriction site may be required" would have been understood as applying to the long-probe technique, but not to the short-probe technique as described.

In contrast, the first and third disadvantages identified in the quotation from the Cohen *et al.* '840 French patent set forth above would have been understood by persons of ordinary skill in the art as applying both to the long-probe technique and to the short-probe technique as described, particularly since the two disadvantages were stated in unconditional terms. Thus it is submitted that the Cohen *et al.* French patent taught that the long-probe technique shared with the short-probe technique a pair of disadvantages; namely, "the temperature conditions are difficult to master to achieve suitable hybridization" and "the nucleic acid is immobilized on a membrane (Southern blot)." Moreover, it is submitted that persons of ordinary skill in the art would have understood from the Cohen *et al.* French patent that the long-probe technique had the two disadvantages of: "the temperature conditions are difficult to master to achieve suitable hybridization" and "the nucleic acid is immobilized on a membrane (Southern blot);" notwithstanding the recognition of such persons, as discussed above in connection with the gene-analysis technique described on page 169 of the Stryer textbook and consistent with the

description of the long-probe technique in the Cohen *et al.* French patent, that the probe of the long-probe technique was used to visualize an electrophoresis pattern from DNA digested by a restriction enzyme in solution and did not discriminate between the two single-nucleotide polymorphisms, but merely marked the location of size-separated fragments on a membrane to which fragments such probes were hybridized according to the technique.

We submit further that the disadvantage of temperature conditions being difficult to master to achieve suitable hybridization and the disadvantage of requiring nucleic acid to be immobilized on a membrane identified by the Cohen *et al.* '840 French patent for the long-probe technique would have been recognized by persons of ordinary skill in the art to apply essentially equally to the method of the Davis *et al.* '372 PCT publication. The method of the Davis *et al.* publication involved irreversibly binding tail-complementary oligonucleotides to a membrane or other substrate at specific locations corresponding to the respective tail sequences of a set of tail-bearing extension primers and applying putative extension products to the substrate under conditions permitting hybridization of the tails of the extension products to respective oligonucleotides complementary to the tails bound to the substrate at specific locations for identifying each location having extension product which bore a label hybridized to the tail-complementary oligonucleotide bound to the substrate at the specific location and so identifying a particular single-nucleotide-polymorphism allele corresponding to the location.

It was asserted in the Office Action on appeal at page 7, lines 10 through 14 that

> [a]ll the teachings of Cohen regarding the disadvantages of immobilized nucleic acids are limited to only methods in which hybridization with the short-probe of long-probe serves to directly detect the presence of a point mutation. Cohen does not teach away from using immobilized nucleic acids for other purposes.

However, the long-probe technique did not require hybridization specificity sufficient to directly detect the presence of a point mutation, yet the Cohen *et al.* '840 French patent disclosed that in the long-probe technique, "the temperature conditions are difficult to master to achieve suitable hybridization," which the Cohen *et al.* patent expressly characterized as a disadvantage of the

technique. Persons of ordinary skill in the art with the Cohen *et al.* patent and the Davis *et al.* publication at hand would have recognized that in the method of the Davis *et al.* publication, the temperature conditions would have been difficult to master to achieve suitable hybridization just as in the long-probe technique, which would have been a disadvantage leading away from the hypothetical combination of the process of the Cohen *et al.* patent with the method of the Davis *et al.* publication proposed by the examiner in the Office Action on appeal.

Importantly, the Cohen *et al.* '840 French patent distinguished both the long-probe technique and the short-probe technique from the method of the patent not only on the basis of temperature conditions being difficult to master to achieve suitable hybridization, but also on the basis of a requirement – specifically characterized in the Cohen *et al.* patent as a disadvantage – to immobilize nucleic acid on a membrane in both the long probe and short probe techniques. The requirement to immobilize nucleic acid on a membrane was a distinguishing feature shared in common by the three previously known techniques distinguished from the method of the Cohen *et al.* patent in the patent specification. In teaching that it was a disadvantage to immobilize nucleic acid on a membrane in both the long-probe technique and the short-probe technique – as well as in the method of the Mundy '127 patent, as discussed below – it is submitted that the Cohen *et al.* '840 French patent taught directly away from the hypothetical combination of the process of the Cohen *et al.* patent with the method of the Davis *et al.* '372 PCT published application proposed in Office Action of 22 May 2007, since the method of the Davis *et al.* published application involved immobilizing nucleic acid on a membrane.

The Mundy '127 patent has been made of record in the subject application and a copy of the patent is included in the evidence appendix accompanying the present brief. In the Mundy '127 patent, the method of the patent for detecting a mutation of a specific nucleotide base in a target nucleic acid chain was distinguished both from the long-probe Southern blot technique discussed above – see column 1, lines 15 through 35 of the Mundy patent – and from the short-probe Southern blot technique of the Conner *et al.* publication – see column 1, lines 36 through 50 of the Mundy patent. However, in embodiments of the method of the Mundy '127 patent

characterized in the patent as preferred, the identity of the mutation would have been determined by detecting the presence or absence of a labeled probe reversibly hybridized to the target nucleic acid irreversibly bound to a nitrocellulose filter. For example, it was disclosed at column 5, lines 6 through 11 of the Mundy patent that single-stranded target chains were preferably immobilized on nitrocellulose. According to the patent, such immobilization could have been

> effected by spotting purified DNA onto nitrocellulose filters and baking at 80° C. to fix the single-stranded target, or possibly by direct processing of cells on nitrocellulose filters.

According to the abstract of the Mundy '127 patent, the method of the patent would have included the step of hybridizing a labeled probe to the target nucleic acid chain to form a hybrid in which one end of the probe would have been positioned adjacent to the specific base to be identified. Regarding the labeled probe, it was disclosed at column 5, lines 36 through 38 of the Mundy patent that the probe could have two sequences, one to hybridize to the target chain and one to carry the label. The abstract of the Mundy '127 patent disclosed that, after the hybrid complex of labeled probe and target nucleic acid chain would have been formed, a digestion-resistant nucleotide derivative such as a thionucleotide would have been added under conditions to cause it to join the end of the probe if it were complementary to the specific base. The resulting hybrid complex would have then been treated with an exonuclease enzyme under conditions such that, if present on the end of the probe, the digestion-resistant nucleotide derivative would have protected the probe from digestion. As disclosed in the abstract of the Mundy patent and at column 2, lines 21 through 23 of the patent, observation of the presence or absence of the probe label attached to the target nucleic-acid chain after the digestion step would have detected the mutation of specific base in the target chain. In embodiments of the method of the Mundy '127 patent characterized as preferred in which the target nucleic-acid chain would have been immobilized on a nitrocellulose filter by spotting and baking, the identity of the mutation would therefore have been determined by observing the presence or absence of a labeled probe reversibly hybridized to the target nucleic acid, which in turn would have been irreversibly bound to the nitrocellulose filter. It is submitted that a person of ordinary skill in the

art would have understood the method of the Mundy '127 patent in embodiments characterized in the patent as preferred, like the long-probe technique and the short-probe technique in spite of differences noted in the Mundy patent itself, to have involved detecting a hybrid nucleic-acid complex immobilized on a membrane, in which a first component of the hybrid complex would have been a nucleic acid effectively irreversibly bound to the membrane by baking or a process of similar effect and a second component of the hybrid complex would have been a labeled oligonucleotide reversibly bound to the first component by hybridization.

It is demonstrated below that the method of the Davis *et al.* '372 PCT published application, which was proposed to be combined with the process of the Cohen *et al.* '840 French patent in the Office Action on appeal, also involved detecting a hybrid nucleic-acid complex immobilized on a membrane, in which a first component of the hybrid complex would have been a nucleic acid effectively irreversibly bound to the membrane by baking or a process of similar effect and a second component of the hybrid complex would have been a labeled oligonucleotide reversibly bound to the first component by hybridization. As pointed out above, the Davis *et al.* published application disclosed a technique for determining the existence or nonexistence of a test nucleotide on a strand of DNA which employed a polymerization agent capable of synthesizing an extension product if there were a match between the test nucleotide on the DNA strand and a nucleotide opposite on an extension primer, but not if there were a mismatch. A single sample of DNA could be tested simultaneously for multiple alleles at a single locus or for a single allele or multiple alleles at multiple loci according to the Davis *et al.* '372 PCT published application by treating the DNA with a plurality of different oligonucleotide primers, each primer being complementary to a different allele and each having a unique oligonucleotide tail. The primers and the DNA would then have been subjected to conditions that would have allowed the primers and DNA to pair and labeled extension products to form if there were a match between a test nucleotide and the opposite nucleotide on the primer, but not if there were a mismatch.

It was disclosed at page 6, lines 7 through 22 and page 21, lines 8 through 12 of the Davis

et al. published application that the presence or absence of a particular extension product could be determined by applying the putative extension products to a substrate such as filter paper, nylon, or nitrocellulose "spotted" at distinct locations with unique oligonucleotides complementary to each of the unique oligonucleotide tails. In Example 4 of the Davis *et al.* '372 PCT published application, a procedure for spotting nylon with oligonucleotides complementary to each of the unique oligonucleotide tails was identified. Specifically, at page 51, lines 17 through 21 of the Davis *et al.* published application, it was disclosed that two poly-complements to two oligonucleotide tails could be bound to discrete locations on a nylon membrane identified by the brand name "ZetaProbe" using conditions recommended by the manufacturer BioRad. Included in the evidence appendix of the present brief is a copy of an instruction manual for "Zeta-Probe® Blotting Membranes" published by Bio-Rad Laboratories of Hercules, California, which had previously been made of record in the subject application. The publication date of the instruction manual is not known. It nonetheless seems of interest to note that Section 2.5 on pages 8 and 9 of the manual describes procedures recommended for DNA dot blotting using Zeta-Probe nylon membranes. Step 7 of the recommended procedure calls UV-crosslinking the DNA to the membrane or vacuum drying the membrane bearing the DNA for 30 minutes at 80° C. Persons of ordinary skill in the art, it is submitted, would have understood such conditions to have led to an essentially irreversible binding of the DNA to the membrane. Whatever the status of the Zeta-Probe manual with respect to availability as of the publication date of the Davis *et al.* '372 PCT published application, the Davis *et al.* published application disclosed at page 8, lines 1 through 5 that the oligonucleotides complementary to each of the unique oligonucleotide tails were preferably "firmly bound" to the substrate and disclosed at page 22, lines 14 through 19 that such oligonucleotides were preferably covalently linked to the substrate.

In the method of the Davis *et al.* '372 PCT published application, to such oligonucleotides firmly bound, covalently linked to a substrate were hybridized the respective complementary oligonucleotide tails of oligonucleotide primers, labeled or not depending on whether there was a match or a mismatch between the 3'end of the corresponding primer at the

site of a test nucleotide on a strand of DNA. The method of the Davis *et al.* published application thus involved detecting a hybrid nucleic-acid complex immobilized on a membrane, in which a first component of the hybrid complex – a unique oligonucleotide complementary to a unique oligonucleotide tail of one of the primers – would have been a nucleic acid effectively irreversibly bound to the membrane by baking or a process of similar effect and a second component of the hybrid complex – a labeled or unlabeled unique oligonucleotide primer with its unique oligonucleotide tail – would have been a labeled oligonucleotide reversibly bound to the first component by hybridization.

At page 6, lines 29 through 33 of the Cohen *et al.* '840 French patent, it was disclosed that a purported advantage of the process of the patent was that the process did not require immobilization of the nucleic acid on a membrane. As may be seen, for example, at page 1, lines 5 through 13, and page 2, lines 8 through 18 of the Cohen *et al.* patent, in the context of the patent, the term "nucleic acid" applied generally to each strand of hybridized DNA or RNA, including probes 150 nucleotides long and shorter probes. Furthermore, at page 3, lines 23 through 29 of the Cohen *et al.* patent, the term "nucleic acid" was applied to a double-stranded hybrid. As noted above, the necessity to immobilize nucleic acid on a membrane was specifically pointed out in the Cohen *et al.* '840 French patent to be a disadvantage shared in common by the previously-known long-probe and short-probe techniques – and the substantially different method of the Mundy '127 patent. See page 3, lines 10 through 17 and page 4, lines 14 through 17 of the Cohen *et al.* patent. It is submitted therefore that the Cohen *et al.* '840 French patent would have directly led persons skilled in the art away from any technique which shared the requirement of immobilization of nucleic acid on a membrane in either single-stranded or double-stranded hybrid form.

Persons of ordinary skill in the art would have recognized, it is submitted, that the multiple-allele/multiple-loci method of the Davis *et al.* '372 PCT published application was just such a technique involving immobilization of nucleic acid on a membrane from which the Cohen *et al.* patent taught away.

As noted above, the Cohen *et al.* '840 French patent drew no distinction between reversibly immobilizing nucleic acid on a membrane and irreversibly immobilizing nucleic acid on a membrane, but declared without qualification on page 6, lines 29 through 33 of the specification that an advantage of the process of the patent was that the process did not require immobilization of the nucleic acid on a membrane. As pointed out above, the term "nucleic acid" was used in the Cohen *et al.* French patent to refer to double-stranded hybrids and to single strands of nucleotides. Each of the three previously known techniques for identifying a single nucleotide mutation distinguished in the Cohen *et al.* patent from the process of the patent in terms of having the disadvantage of requiring nucleic acid immobilized on a membrane would, it is submitted, have been expected by persons of ordinary skill in the art generally to have involved detecting a hybrid nucleic-acid complex immobilized on a membrane, in which a first component of the hybrid complex was a nucleic acid effectively irreversibly bound to the membrane and a second component of the hybrid complex was a labeled oligonucleotide of some sort reversibly bound to the first component by hybridization. Moreover, persons of ordinary skill in the art would have recognized, it is submitted, that the method of the Davis *et al.* '372 PCT published application likewise involved detecting a hybrid nucleic-acid complex immobilized on a membrane in which a first component of the hybrid complex was a nucleic acid effectively irreversibly bound to the membrane and a second component of the hybrid complex was a labeled oligonucleotide reversibly bound to the first component by hybridization and therefore such persons would have appreciated that the method of the Davis *et al.* published application necessarily entailed a feature which the Cohen *et al.* '840 French patent characterized specifically as a disadvantage not shared by the process disclosed in the Cohen *et al.* patent. Persons of ordinary skill in the art would thus have deemed it in no way obvious to combine the process of the Cohen *et al.* patent with the method of the Davis *et al.* '372 PCT published application as proposed in the Office Action of 22 May 2007.

For the reasons set forth above, it is submitted that, assuming for the sake of argument only that the hypothetical combination of the single-base-identification process of the Cohen *et al.* '840 French patent with the multiple-allele/multiple-loci method of the Davis *et al.*

'372 PCT published application proposed in the Office Action on appeal would even have occurred to a person of ordinary skill on the art as of the effective date of the subject application, such a person would have recognized that the method of the Davis *et al.* '372 PCT published application involved immobilizing nucleic acid on a substrate and that the method would therefore have effectively shared the disadvantage of three previously known methods requiring immobilizing nucleic acid on a membrane specifically pointed out in the Cohen *et al.* patent and would have recognized further that the method of the Davis *et al.* '372 PCT published application would have shared the disadvantage of temperature conditions being difficult to master to achieve suitable hybridization pointed out in the Cohen *et al.* French patent for the long-probe Southern blot technique. It is submitted therefore that a person of ordinary skill in the art would not have attempted to combine the single-base-identification process of the Cohen *et al.* '840 French patent with the multiple-allele/multiple-loci method of the Davis *et al.* '372 PCT published application in view of the teachings in the Cohen *et al.* patent directly away from such the hypothetical combination.

It is therefore submitted that the Cohen *et al.* '840 French patent (or, alternatively, the Cohen *et al.* '883 published European application) considered alone or in combination with the Davis *et al.* '372 PCT published application would not have disclosed or suggested the method of independent claim 64 of the subject application to a person of ordinary skill in the art as of the effective filing date of the application. The rejection under 35 U.S.C. § 103(a) of independent claim 64 as unpatentable over Cohen *et al.* '840 French patent (or the Cohen *et al.* '883 published European application) in view of the Davis *et al.* '372 PCT published application was without justification, it is submitted, and should be reversed.

Each of claims 66, 67, 69, and 70 respectively depends directly or indirectly on independent claim 64 and consequently incorporates the limitations of independent claim 64 by reference. The reasoning set forth above concerning distinctions between the Cohen *et al.* '840 French patent considered alone or in combination with the Davis *et al.* '372 PCT published application and the method of independent claim 64 therefore applies with equal force with

respect to dependent claims 66, 67, 69, and 70. Consequently, it is submitted that the Cohen *et al.* '840 French patent considered alone or in combination with the Davis *et al.* '372 PCT published application would have neither disclosed nor in any way suggested the subject matter of claims 66, 67, 69, and 70 to a person of ordinary skill in the art as of the effective filing date of the subject application. It is submitted, therefore, that the rejection under 35 U.S.C. § 103(a) of dependent claims 66, 67, 69, and 70 of the subject application as unpatentable over the Cohen *et al.* '840 French patent (or the Cohen *et al.* '883 published European application) in view of the Davis *et al.* '372 PCT published application was without justification and should be reversed.

To summarize, for the reasons set forth above, it is submitted that the final rejection under 35 U.S.C. § 103(a) of claims 64, 66, 67, 69, and 70 of the subject application as unpatentable over the Cohen *et al.* '840 French patent (or the Cohen *et al.* '883 published European application) in view of the Davis *et al.* '372 PCT published application was unwarranted and should be reversed.

VII.*c.*) The Cohen *et al.* '840 French Patent in View of the Davis *et al.* '372 PCT Published Application and the Prober *et al.* '666 Patent

VII.*c.*1.) Summary of Office Action on Appeal with Respect to Final Rejection Under 35 U.S.C. § 103(a) of Claim 68 as Unpatentable over the Cohen *et al.* '840 French Patent in View of the Davis *et al.* '372 PCT Published Application and the Prober *et al.* '666 Patent

Claim 68 was finally rejected in the Office Action of 22 May 2007 under 35 U.S.C. § 103(a) as unpatentable over the Cohen *et al.* '883 published European application or the Cohen *et al.* '840 French patent, each in view of the Davis *et al.* '372 PCT published application and United States patent No. 5,332,666 to Prober *et al.* ("the Prober *et al.* '666 patent"). It was conceded in the Office Action of 22 May 2007 that the hypothetical combination of the Cohen *et al.* patent and the Davis *et al.* published application proposed in the Office Action did not disclose using a terminator that comprised arabinoside

triphosphate. It was asserted that the Prober *et al.* '666 patent disclosed that a terminator may contain an arabinose as the sugar group. It was asserted in the 22 May 2007 Office Action that it would have been obvious to one of ordinary skill in the art to have modified the method of the Cohen *et al.* '840 French patent so as to have a terminator comprising an arabinoside triphosphate.

VII.*c*.2.) The Prober *et al.* '666 Patent Does Not Overcome the Teachings of the Cohen *et al.* '840 French Patent Away From the Proposed Hypothetical Combination of the Method of the Cohen *et al.* Patent and the Method of the Davis *et al.* '372 PCT Published Application

The Prober '666 patent in no way overcomes the teachings of the Cohen *et al.* '840 French patent against the hypothetical combination of the single-base-identification process of the '840 French patent with the multiple-allele/multiple-loci method of the Davis *et al.* '372 PCT published application proposed in the Office Action on appeal discussed in the subsection VII.*b*.2.) above and consequently the reasoning of the subsection VII.*b*.2.) above with regard to independent claim 64 applies equally with respect to the final rejection of claim 68 in the Office Action of 22 May 2007 under 35 U.S.C. § 103(a) as unpatentable over the Cohen *et al.* '840 French patent in view of the Davis *et al.* '372 PCT published application and the Prober *et al.* '666 patent, since claim 68 depends on claim 64. It is submitted that the final rejection in the Office Action of 22 May 2007 of claim 68 of the subject application as amended under 35 U.S.C. § 103(a) as unpatentable over the Cohen *et al.* '840 French patent or the Cohen *et al.* '883 published European application in view of the Davis *et al.* '372 PCT published application and the Prober *et al.* '666 patent was unjustified and should be reversed.

VII.*d.*) The Cohen *et al.* '840 French Patent in View of the Davis *et al.* '372 PCT Published Application and the Tabor *et al.* '020 Patent

VII.*d.*1.) Summary of Office Action on Appeal with Respect to Final Rejection Under 35 U.S.C. § 103(a) of Claim 71 as Unpatentable over the Cohen *et al.* '840 French Patent in View of the Davis *et al.* '372 PCT Published Application and the Tabor *et al.* '020 Patent

Claim 71 was finally rejected in the Office Action of 22 May 2007 under 35 U.S.C. § 103(a) as unpatentable over the Cohen *et al.* '883 published European application or the Cohen *et al.* '840 French patent, each in view of the Davis *et al.* '372 PCT published application and United States patent No. 4,962,020 to Tabor *et al.* ("the Tabor *et al.* '020 patent"). In the Office Action it was stated that the hypothetical combination of the Cohen *et al.* '840 French patent and the Davis *et al.* '372 PCT published application proposed in the Office Action did not disclose including pyrophosphatase in the primer extension medium. It was asserted that the Tabor *et al.* '020 patent disclosed including pyrophosphatase in primer extension reactions to remove pyrophosphate which builds up in such reactions. The Tabor *et al.* '020 patent assertedly disclosed that, in the presence of pyrophosphate, DNA polymerase adds pyrophosphate to the 3' terminal nucleotide, assertedly causing release of dideoxynucleoside 5'-triphosphates, removing the block at the 3' terminus. It was asserted that in the Office Action of 22 May 2007 that it would have been obvious to one of ordinary skill in the art to have modified the method of the Cohen *et al.* '840 French patent so as to have included pyrophosphatase in the reaction medium assertedly to eliminate pyrophosphorolysis activity of DNA polymerase assertedly to reduce the probability that a labeled terminator would be removed and unlabeled dideoxynucleotides would be released into the reaction medium.

VII.*d*.2.) The Tabor *et al.* '020 Patent Does Not Overcome the Teachings of the Cohen *et al.* '840 French Patent Away From the Proposed Hypothetical Combination of the Method of the Cohen *et al.* Patent and the Method of the Davis *et al.* '372 PCT Published Application

As in the case of the Prober *et al.* '666 patent discussed in the preceding subsection, the Tabor *et al.* '020 patent in no way overcomes the teachings of the Cohen *et al.* '840 French patent against the hypothetical combination of the single-base-identification process of the '840 French patent with the multiple-allele/multiple-loci method of the Davis *et al.* '372 PCT published application proposed in the Office Action of 22 May 2007 discussed above and consequently the reasoning of the subsection VII.*b*.2.) above with regard to independent claim 64 applies equally with respect to the final rejection of claim 71 in the Office Action of 22 May 2007 under 35 U.S.C. § 103(a) as unpatentable over the Cohen *et al.* '840 French patent in view of the Davis *et al.* '372 PCT published application and the Tabor *et al.* '020 patent, since claim 71 depends on claim 64. It is submitted that the rejection in the Office Action of 22 May 2007 of claim 71 of the subject application as amended under 35 U.S.C. § 103(a) as unpatentable over the Cohen *et al.* '840 French patent or the Cohen *et al.* '883 published European application in view of the Davis *et al.* '372 PCT published application and the Tabor *et al.* '020 patent was unwarranted and should be reversed.

VIII. Conclusion

For the reasons set forth above, it is submitted that the claims of the subject application are patentable over the art of record considered alone or in any combination. Reversal of the final rejections of the Office Action of 22 May 2007 and allowance of the application is therefore earnestly solicited.

Respectfully submitted,

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Claims Appendix

Claim 64. A method of determining the identity of one or more nucleotide bases at a plurality of specific positions in one or more nucleic acid molecules of interest, comprising:

(a) treating a sample comprising the one or more nucleic acid molecules of interest, if the nucleic acid molecules of interest comprise double-stranded nucleic acid, so as to obtain unpaired nucleotide bases spanning the specific positions, or directly employing a sample comprising the one or more nucleic acid molecules of interest in step (b) if the nucleic acid is singlestranded;

(b) contacting the sample from step (a) with a plurality of different oligonucleotide primers, wherein:

(i) each such different oligonucleotide primer hybridizes, under high stringency hybridization conditions, to a corresponding different stretch of nucleotide bases present in the nucleic acid molecules of interest which is immediately adjacent to the specific position of a nucleotide base to be identified with that oligonucleotide primer, so as to form a duplex such that the nucleotide base to be identified is the first unpaired base of the nucleic acid molecule of interest immediately downstream of the 3' end of the oligonucleotide primer; and

(ii) each different oligonucleotide primer comprises a corresponding different affinity moiety, the oligonucleotide primer comprising the affinity moiety being capable of hybridizing with a nucleic-acid template and undergoing a nucleic acid template-dependent primer extension reaction with terminators of a terminator reagent, the affinity moiety permitting affinity separation of the extended oligonucleotide primer from the terminator reagent;

(c) contacting the duplexes from step (b) with a terminator reagent free of dATP, dCTP, dGTP, and dTTP and comprising four different terminators of a nucleic acid

template-dependent primer extension reaction, each terminator comprising a different detectable label corresponding to the terminator, wherein one of the terminators is complementary to a nucleotide base to be identified by each of the oligonucleotide primers, wherein the contacting is carried out in a primer-extension reaction medium under conditions sufficient to permit a templatedependent primer extension reaction which incorporates the complementary terminator onto the 3' end of each of the different oligonucleotide primers to thereby extend the 3' end of each of the oligonucleotide primers by one terminator;

(d) affinity separating the respective extended oligonucleotide primers from primer-extension reaction medium by causing each of the extended oligonucleotide primers to contact an affinity group attached to a solid support, such affinity group being complementary to the affinity moiety incorporated in the oligonucleotide primer; and

(e) determining the presence and identity of the nucleotide base at each of the respective specific positions in the one or more nucleic acid molecules of interest by detecting the detectable label of the terminator incorporated at the 3' end of each of the affinity separated extended oligonucleotide primers.

Claim 66. A method according to claim 64, wherein the terminators comprise dideoxynucleotides.

Claim 67. A method according to claim 66, wherein the terminators comprise one or more of ddATP, ddCTP, ddGTP, ddTTP or ddUTP.

Claim 68. A method according to claim 64, wherein the terminators comprise arabinoside triphosphates.

Claim 69. A method according to claim 64, wherein each of the detectable labels is an isotopically labeled moiety, a chromophore, a fluorophore, a protein moiety, or a moiety to which an isotopically labeled moiety, a chromophore, a fluorophore, or a protein moiety can be attached.

Claim 70. A method according to claim 64, wherein each of the different detectable labels is a different fluorophore.

Claim 71. A method according to claim 64, wherein the primer-extension reaction medium further comprises pyrophosphatase.

Evidence Appendix

(1) SOUTHERN, E. M., Detection of Specific Sequences Among DNA Fragments Separated by Gel Electrophoresis, *Journal of Molecular Biology*, 1975, pages 503 through 517, volume 98 ("the Southern publication") - entered with Information Disclosure Statement of 26 June 2006;

(2) CONNER, B. J. *et al.*, Detection of sickle cell β^S-globin allele by hybridization with synthetic oligonucleotides, *Proceedings of the National Academy of Sciences USA*, January 1983, pages 278 through 282, volume 80, ("the Conner *et al.* publication") - entered with Information Disclosure Statement of 26 June 2006;

(3) United States patent 4,656,127 to MUNDY, C. R. ("the Mundy '127 patent") - entered with Information Disclosure Statement of 8 June 1999;

(4) STRYER, L. *Biochemistry*, third edition, 1988, page 169, W.H. Freeman and Company, New York ("the Stryer textbook") - entered with Information Disclosure Statement of 9 March 2007;

(5) *Zeta-Probe® Blotting Membranes, Instruction Manual*, (LIT234 Rev C), undated, 29 pages, Bio-Rad Laboratories, Hercules, California ("the Zeta-Probe manual") - entered with Information Disclosure Statement of 26 June 2006

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Detection of Specific Sequences Among DNA Fragments Separated by Gel Electrophoresis

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This paper describes a method of transferring fragments of DNA from agarose gels to cellulose nitrate filters. The fragments can then be hybridized to radioactive RNA and hybrids detected by radioautography or fluorography. The method is illustrated by analyses of restriction fragments complementary to ribosomal RNAs from *Escherichia coli* and *Xenopus laevis*, and from several mammals.

1. Introduction

Since Smith and his colleagues (Smith & Wilcox, 1970; Kelly & Smith, 1970) showed that a restriction endonuclease from *Haemophilus influenzae* makes double-stranded breaks at specific sequences in DNA, this enzyme and others with similar properties have been used increasingly for studying the structure of DNA. Fragments produced by the enzymes can be separated with high resolution by electrophoresis in agarose or polyacrylamide gels. For studies of sequences in the DNA that are transcribed into RNA, it would clearly be helpful to have a method of detecting fragments in the gel that are complementary to a given RNA. This can be done by slicing the gel, eluting the DNA and hybridizing to RNA either in solution, or after binding the DNA to filters. The method is time consuming and inevitably leads to some loss in the resolving power of gel electrophoresis. This paper describes a method for transferring fragments of DNA from strips of agarose gel to strips of cellulose nitrate. After hybridization to radioactive RNA, the fragments in the DNA that contain transcribed sequences can be detected as sharp bands by radioautography or fluorography of the cellulose nitrate strip. The method has the advantages that it retains the high resolving power of the gel, it is economical of RNA and cellulose nitrate filters, and several electrophoretograms can be hybridized in one day. The main disadvantage is that fragments of 500 nucleotide pairs or less give low yields of hybrid and such fragments will be under-represented or even missing from the analysis.

2. Materials, Methods and Results

(a) *Restriction endonucleases*

EcoRI prepared according to the method of Yoshimuri (1971) was a gift of K. Murray. HaeIII prepared by a modification of the method of Roberts (unpublished data) was a gift of H. J. Cooke.

(b) *Gel electrophoresis*

Gels were cast between glass plates (de Wachter & Fiers, 1971). The plates were separated by Perspex side pieces 3 mm thick and along one edge was placed a "comb" of Perspex, which moulded the sample wells in the gel. The Perspex pieces were sealed to the glass plates with silicone grease and the plates clamped together with Bulldog clips. The assembly was stood with the comb along the lower edge. Agarose solution (Sigma electrophoresis grade agarose) was prepared by dissolving the appropriate weight in boiling electrophoresis buffer (E buffer of Loening, 1969). The solution was cooled to 60 to 70°C and poured into the assembly, where it was allowed to set for at least an hour. The assembly was then inverted, the comb removed and the wells filled with electrophoresis buffer. Samples made 5% with glycerol were loaded from a drawn-out capillary by inserting the tip below the surface and blowing gently. Electrophoresis buffer was layered carefully to fill the remaining space and a filter-paper wick inserted between the glass plates along the top edge. The lower end of the assembly was immersed in a tray of electrophoresis buffer containing the platinum anode, and the paper wick dipped into a similar cathode compartment. Electrophoresis was at 1·0 to 1·5 mA/cm width of gel for a period of about 18 h. Bromophenol blue marker travels about 3/4 the length of the gel under these conditions, but it should be noted that small DNA fragments move ahead of the bromophenol blue, especially in dilute gels. Cylindrical gels were cast in Perspex tubes 9 mm i.d. and either 12 or 24 cm long. These were run at 3 to 5 mA/tube in standard gel electrophoresis equipment.

Vol. 98
1975

Dr J. Spiers donated ribosomal DNA that had been purified on actinomycin/caesium chloride gradients from DNA made from the pooled blood of several animals, and also ^{3}H-labelled 18 S and 28 S RNAs prepared from cultured *Xenopus laevis* kidney cells. *Escherichia coli* DNA was prepared by Marmur's (1961) procedure from strain MRE600. ^{32}P-labelled *E. coli* RNA was prepared from cells grown in low phosphate medium with ^{32}Pi at a concentration of 50 μCi/ml and fractionated by electrophoresis on 10% acrylamide gels. ^{32}P-labelled rat DNA was a gift of M. S. Campo. DNA from human placenta was a gift of H. J. Cooke, DNA from rat liver was a gift of A. R. Mitchell, DNA from mouse and rabbit livers were gifts of M. White. Calf thymus DNA was purchased from Sigma Biochemicals. For digestion with restriction endonucleases, the DNAs were dissolved in water to a concentration of approximately 1 mg/ml. One-tenth volume of the appropriate buffer was added and sufficient enzyme to give a complete digestion overnight at 37°C. Enzyme activity was checked on phage λ DNA and digests of this DNA were also used as size markers in gel electrophoresis, using the values given by Thomas & Davis (1975).

(c) *Method of transfer*

This section describes the method finally adopted: preliminary experiments and controls are described in later sections.

After electrophoresis, the gel is immersed for 1 to 2 h in electrophoresis buffer containing ethidium bromide (0·5 μg/ml), and photographed in ultraviolet light (254 nm) with a red filter on the camera. A rule laid alongside the gel aids in matching the photograph of the fluorescence of the DNA to the final radioautograph of the hybrids. Strips to be used for transfer from flat gels are cut from the gel using a flamed blade. The strips should be 0·5 cm to 1 cm wide and normally extend from the origin to the

anode end of the gel. The gels used in this laboratory are 3 mm thick, and the length from the origin to the anode end is 18 cm but the method can be adapted to gels with different dimensions and to cylindrical gels. Strips of gel are then transferred to measuring cylinders containing 1·5 M-NaCl, 0·5 M-NaOH for 15 min and this solution is then replaced by 3 M-NaCl, 0·5 M-Tris·HCl (pH 7) and the gel is left for a further 15 min. The depth of liquid in the cylinders should be greater than the length of the gel strips and the cylinders should be inverted from time to time. For cylindrical gels (9 mm diam.), the times required for denaturation and neutralization are 30 and 90 min. Each gel transfer requires:

One piece of thick filter paper 20 cm × 18 cm, soaked in 20 × SSC (SSC is 0·15 M-NaCl, 0·015 M-sodium citrate).

Two pieces of thick filter paper 2 cm × 18 cm soaked in 2 × SSC.

One strip of cellulose nitrate filter (e.g. Millipore 25 HAWP), 2·2 cm × 18 cm, soaked in 2 × SSC. These strips are immersed first by floating them on the surface of the solution; otherwise air is trapped in patches, which leads to uneven transfer.

Three pieces of glass or Perspex, 5 cm × 20 cm and the same thickness as the gel.

Four or five pieces of thick, dry filter paper, 10 cm × 18 cm.

Transfer of the denatured DNA fragments is carried out as follows.

The large filter paper soaked in 20 × SSC is laid on a glass or plastic surface, care being taken to avoid trapping air bubbles below the paper. 20 × SSC is poured on so that the surface is glistening wet. One of the glass or Perspex sheets is laid on top of the wet paper. The gel strip is taken from the neutralizing solution and laid parallel to the glass or Perspex sheet, 2 to 3 mm away from it. The second glass or Perspex sheet is laid 2 to 3 mm away from the other side of the gel (Fig. 1(a)). The cellulose nitrate strip is then laid on top of the gel with its edges resting on the sheets of Perspex or glass, so that it bridges the two air spaces (Fig. 1(b)). The two narrow pieces of filter paper, moistened with 2 × SSC are laid with their edges overlapping the cellulose nitrate strip by about 5 mm (Fig. 1(c)) and the dry filter paper is then placed on top of these (Fig. 1(d)).

For cylindrical gels, the arrangement is similar, but in this case, the Perspex that supports the Millipore filter may be in contact with the gel because an air space is retained over the top of the gel. Several cylindrical gels can be transferred at the same time using the apparatus shown in Fig. 2 and similar arrangements can be used for flat gels.

20 × SSC passes through the gel drawn by the dry filter paper and carries the DNA, which becomes trapped in the cellulose nitrate. The minimum time required for complete transfer has not been measured: it depends on the size of the fragments and probably also depends on the gel concentration. A period of 3 h is enough to transfer completely all HaeIII fragments of *E. coli* DNA from 2% agarose gels 3 mm thick. But even after 20 h, transfer of large EcoRI fragments of mouse DNA from 9 mm diam. cylindrical gels is not complete. DNA remaining in the gel can be seen by the fluorescence of the ethidium bromide, which is not completely removed during treatment of the gel. During the period of the transfer, it is necessary occasionally to add more 20 × SSC to the bottom sheet of filter paper. If the paper dries too much, the gel shrinks against the cellulose nitrate strip and liquid contact is broken. The paper may be flooded, but care must be taken that liquid does not fill the air spaces between the gel and the side-pieces and soak the paper, bypassing the gel. It may be found convenient to leave the cellulose nitrate in position overnight: if the supply of

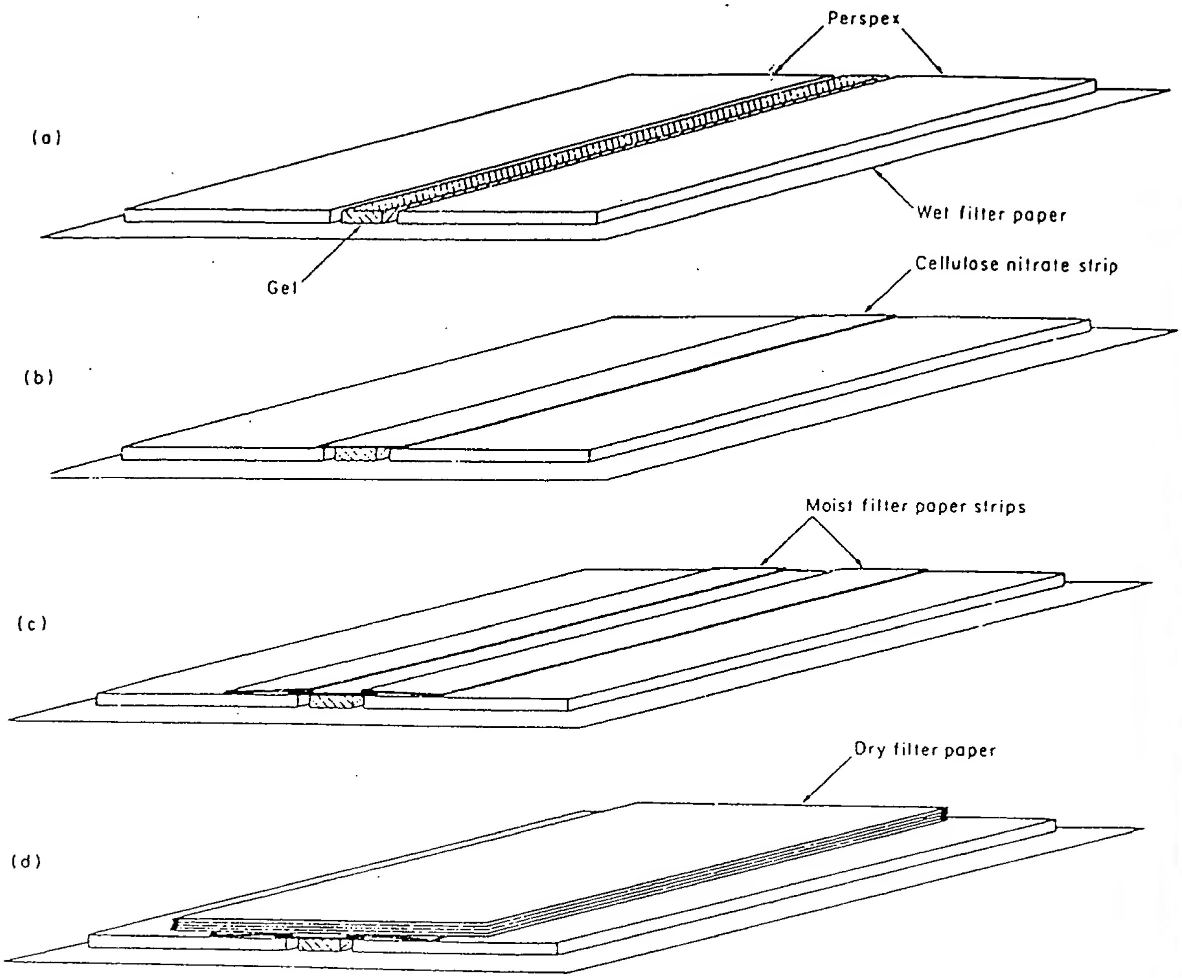

FIG. 1. Steps in the procedure for transferring DNA from agarose gels to cellulose nitrate strips.

20 × SSC has dried up it will be found that the gel has shrunk against the cellulose nitrate, but this does not impair the transfer. At the end of the transfer period the cellulose nitrate strip is lifted carefully so that the gel remains attached to its underside. It is turned over and the outline of the gel marked in pencil by a series of dots. The gel is peeled off the cellulose nitrate, the area of contact cut out with a flamed blade, and immersed in 2 × SSC for 10 to 20 min. The strip is then baked in a vacuum oven at 80°C for 2 h.

(d) *Hybridization*

Radioactive RNAs are usually available in small quantities only and it is important to keep the volume of the solution used for hybridization as small as possible so that the RNA has a reasonable concentration. Two procedures can be used for hybridizing the cellulose nitrate strips after transferring the restriction fragments.

The procedure that uses the smallest volume is carried out by moistening the strip in hybridization mixture and then immersing it in paraffin oil. A drop of RNA solution (0·3 ml for a strip 1 cm × 18 cm) is placed on a plastic sheet. One end of the

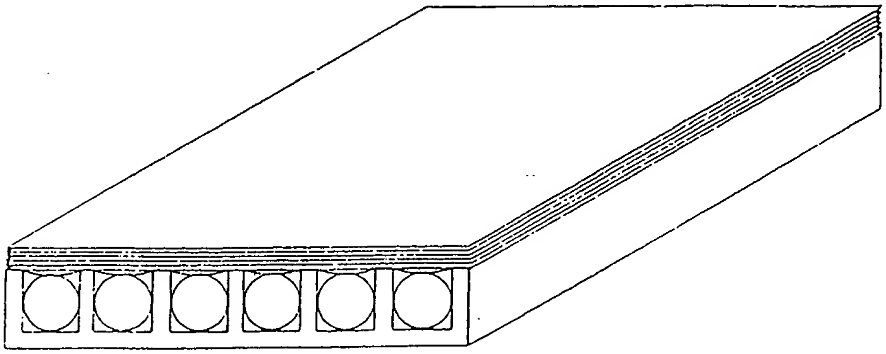

FIG. 2. Apparatus for transferring DNA from a number of cylindrical gels.

The apparatus is constructed of Perspex. The uprights which separate the gels and support the sheet of cellulose nitrate should be about 0·5 mm higher than the diameter of the gels, so that the cellulose nitrate sheet dips down to touch the gel. Thus an air gap is left between the cellulose nitrate sheet and the filter paper, above the line of contact between the gel and cellulose nitrate sheet. The apparatus is laid in a shallow tray containing 20 × SSC and the gels are then inserted into the troughs, care being taken to avoid trapping air bubbles beneath the gel. The cellulose nitrate sheet, wet with 2 × SSC, is laid over the gels and one piece of wet filter paper is laid over this. A stack of dry filter paper is then placed over the whole assembly. If necessary, a glass plate can be used to weigh down the filter papers. The depth of 20 × SSC in the tray should be enough to cover the lower part of the gels, but not so much that the air space between the Perspex and the cellulose nitrate becomes flooded.

cellulose nitrate strip is floated on the drop and when liquid is seen to soak through, the strip is drawn slowly over the surface of the drop. When it is completely wetted from one side, it is turned over and any remaining liquid is used to wet the other side. The strip is then immersed in paraffin oil saturated with the hybridization solution at the hybridization temperature. It should be borne in mind that baking the strip in 2 × SSC introduces salt, which must be taken into account when deciding on a solvent for the RNA if this method of hybridization is used. For example, if hybridization is to be carried out in 6 × SSC the RNA should be dissolved in 4 × SSC. Though this method can give good results (see Plate I) it often leads to high and uneven background. Kourilsky *et al.* (1974) found that this problem is removed if the hybridization is carried out in 2 × SSC, 40% formamide at 40°C. I have not tried this method, because this solvent removed DNA from the filters (see later section). It may well be the best method for hybridization to large fragments. I have found it convenient to carry out the hybridization in a vessel designed to hold the strip in a small volume of liquid.

The vessel (Fig. 3), which is easily made from Perspex, has internal dimensions of 0·8 mm deep by 2 cm high and about 1 cm longer than the strip to be hybridized. The vessel is filled with the solvent to be used for hybridization and the strip is fed in through the narrow opening in the top. The solvent is then drained off and the RNA solution introduced. Around 1 ml of solution is needed for a strip 1 cm × 18 cm. The wide sheets of cellulose nitrate used for transferring several gels (e.g. using the apparatus shown in Fig. 2) are too wide to be hybridized in this type of vessel. They can be hybridized in a small volume by wrapping them around a cylinder of Perspex, which is then inserted into a close-fitting tube. In this way, it is possible to hybridize a sheet 24 cm × 8 cm with about 4 ml of solution. If hybridization is carried out in a water-bath, it is not necessary to seal the top of the vessel provided the water-bath

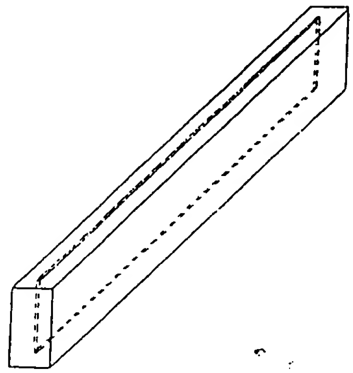

Fig. 3. Vessel used for hybridization of narrow strips.

itself is covered. The liquid in the vessel evaporates very slowly and can be replenished by small additions of water. A further advantage of this method of hybridization is that the RNA can be recovered and used again.

The period allowed for hybridization depends on the RNA concentration, its sequence complexity, its purity, and on the conditions of hybridization (see for example Bishop, 1972). After the appropriate period, strips are removed from the solution or paraffin oil, blotted between sheets of filter paper and washed, with stirring, for 20 to 30 min in a large volume of the hybridization solvent at the hybridization temperature. If the background is high, they may then be treated with a solution of RNAase A (20 μg/ml in 2 $\times$ SSC for 30 min at 20°C). After a final rinse in 2 $\times$ SSC they are dried in air.

So far the method has been tested with ^{32}P, ^{3}H, ^{35}S and ^{125}I-labelled RNAs. [^{32}P]RNAs have been detected by radioautography. For this the cellulose nitrate strips are laid on X-ray film and flattened against it with light pressure. ^{3}H, ^{125}I, ^{35}S and ^{14}C may be detected by fluorography. The cellulose nitrate strip is dipped through a solution of PPO in toluene (20%, w/v) dried in air, laid against X-ray film (Kodak RP-Royal Xomat) and kept at -70°C.

(e) *Completeness of transfer and retention of DNA*

Preliminary experiments showed that loading of DNA on to cellulose nitrate filters in 6 $\times$ SSC, conditions widely used in hybridization work, did not give complete retention of small fragments and a systematic study was made of the effect of salt concentration on retention. ^{3}H-labelled *X. laevis* DNA was sonicated to a single-strand molecular weight of 10^4 and denatured by boiling in 0·1 $\times$ SSC. Samples were made up to various salt concentrations and 0·1-ml portions of these solutions were pipetted on to cellulose nitrate filters, previously moistened with 2 $\times$ SSC, which were resting on glass-fibre filters. The solution that passed through the cellulose nitrate filter was thus collected in the glass-fibre filter. Both filters were then immersed in 5% trichloroacetic acid for 10 min, dried for 30 min in a vacuum oven at 80°C, and counted. It can be seen (Fig. 4) that the fraction of DNA retained by the cellulose nitrate increases with the salt concentration, and at concentrations above 10 $\times$ SSC the DNA is almost completely retained.

Losses of DNA at various stages of the transfer procedure were measured using ^{32}P-labelled *E. coli* DNA. The DNA was digested with EcoRI to give fragments in

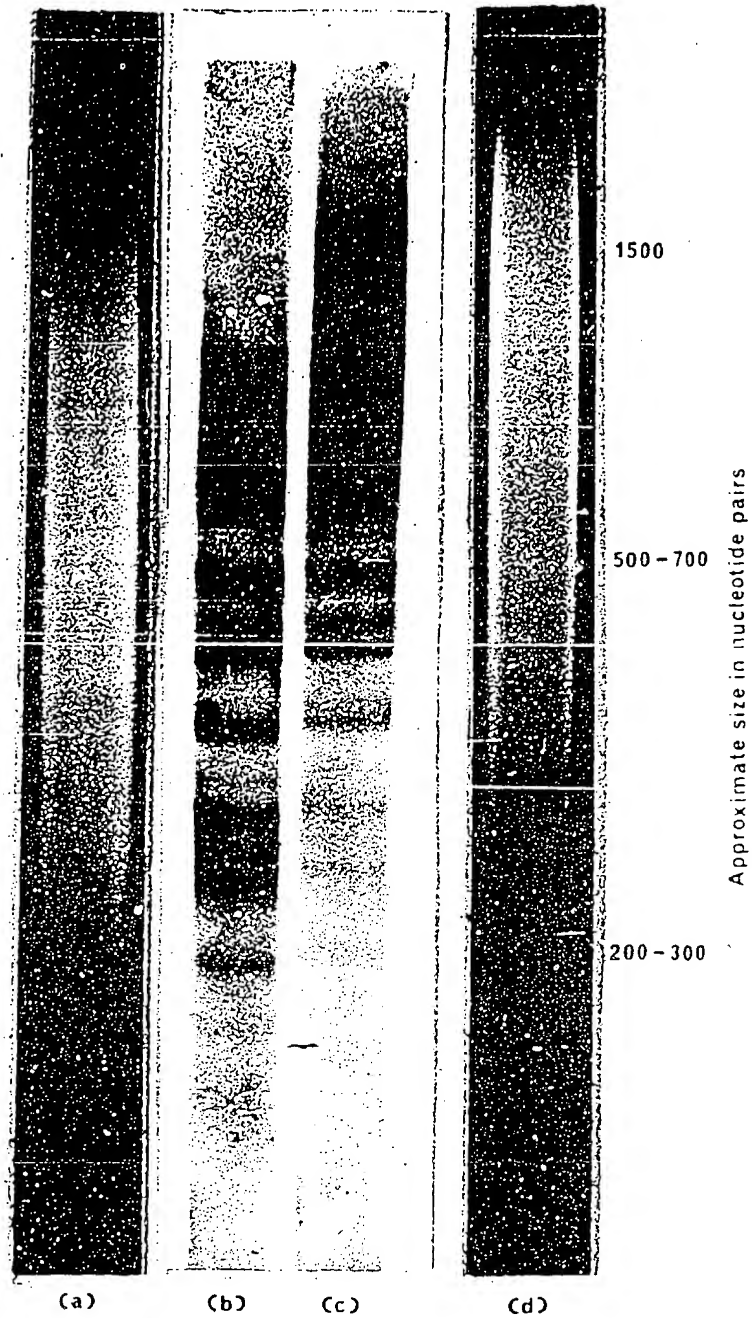

PLATE I. HaeIII digest of *E. coli* MRE600 DNA analyzed by electrophoresis on 2% agarose gel. DNA was then transferred to cellulose nitrate and hybridized with ^{32}P-labelled, high molecular weight RNA. (a) and (d) Photographs of ethidium bromide fluorescence. (b) and (c) Radioautographs of hybrids.

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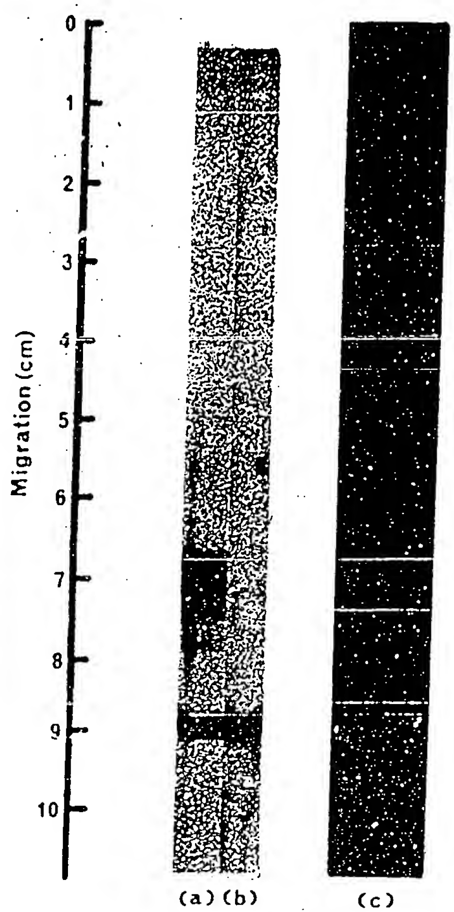

PLATE II. EcoRI digest of purified *X. laevis* ribosomal DNA analyzed by electrophoresis on 1% agarose gel. DNA was transferred to a cellulose nitrate strip, which was then cut longitudinally in two. The left-hand side was hybridized to 18 S RNA and the right-hand side to 28 S RNA (spec. act. of RNAs, $1 \cdot 5 \times 10^6$ c.p.m. per μg). Hybridization was done in $1 \times$ SSC at 65°C using the vessel shown in Fig. 3. A large excess of cold 28 S RNA was added to the labelled 18 S RNA to compete out any 28 S contamination. After hybridization, the strips were washed in $1 \times$ SSC at 65°C for 1·5 h, and dried. They were then dipped through a solution of PPO in toluene (20%, w/v) dried in air and placed against Kodak RP Royal X-ray film at −70°C for 2 months. Photograph of ethidium bromide fluorescence (c). Fluorograph of 18 S hybrids (a). Fluorograph of 28 S hybrids (b).

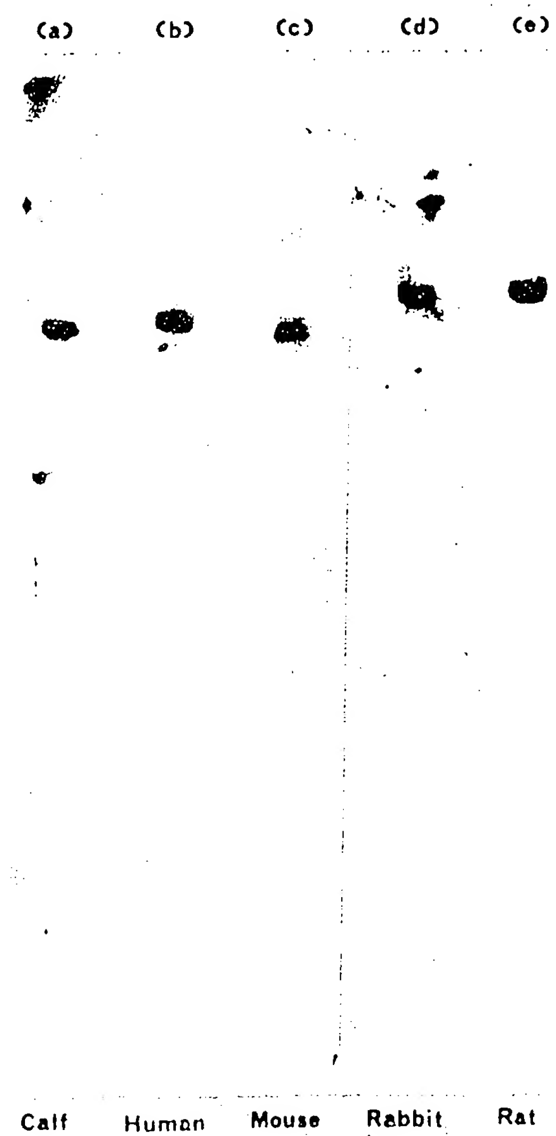

PLATE III. EcoRI digests of five mammalian DNAs, hybridized to 28 S RNA. Calf (a), human (b), mouse (c), rabbit (d) and rat (e) DNAs were digested to completion with EcoRI and separated by electrophoresis on 1% agarose gels (9mm × 12 cm, approx. 40 μg DNA per tube, 3 mA/tube for 16 h). The gels were pretreated as usual and the DNA fragments transferred to a single sheet of cellulose nitrate filter (12 cm × 8 cm) using the apparatus shown in Fig. 2. The top end of each gel was carefully aligned with one edge of the cellulose nitrate sheet. After 20 h, traces of DNA could still be seen, by ethidium bromide fluorescence, in the high molecular weight region of the gel. The filter was hybridized with 28 S RNA and radioautographed as described in the legend to Fig. 8.

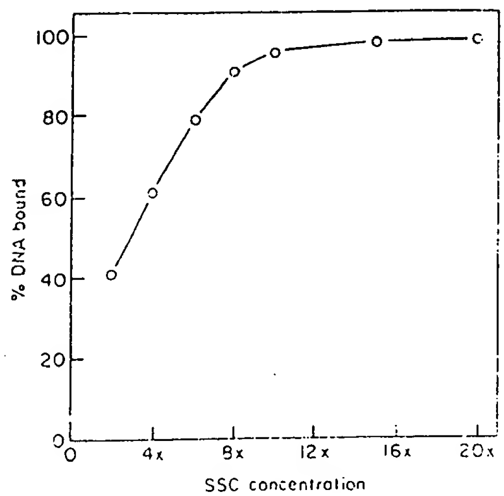

FIG. 4. Effect of salt concentration on efficiency of binding sonicated DNA to cellulose nitrate filters.

the large size range and with HaeIII to give small fragments. The fragments were then separated on a flat 1% agarose gel and transferred in the usual way. The solutions, the gel and the cellulose nitrate strip were counted. It can be seen (Table 1) that, whereas a small proportion of the DNA is leached out into the solutions during denaturation and neutralization, only traces remain in the gel after transfer.

TABLE 1

Losses of DNA at stages of the procedure

	EcoRI fragments	HaeIII fragments
	DNA lost (%)	
Denaturing solution	2·1	4·8
Neutralizing solution	1·3	4·4
Remaining in gel after transfer	0·21	0·31

Two samples of *E. coli* DNA (0·1 μg; spec. act. approx. 10^6 c.p.m. per μg) were digested with EcoRI and HaeIII. The fragments were separated by electrophoresis on 1% gels in 1-cm wide slots, and then transferred to cellulose nitrate strips as described in Materials and Methods. The transfer was left overnight. The radioactivity leached out of the gel by the denaturing and neutralizing solutions, that remaining in the gel, and that which had been trapped on the cellulose nitrate filter were measured in a liquid scintillation counter (Cerenkov radiation).

(f) *Effect of DNA size on yield of hybrid*

Melli & Bishop (1970) have shown that hybridization by the filter method gives low yields with low molecular weight DNA. Their results were obtained using a single set of hybridization conditions and it seemed possible that losses might be reduced by using high salt concentrations. The effect of salt concentration on loss of

DNA from the filters was examined by loading filters with radioactive *X. laevis* DNA, single-strand molecular weight about 10^4, and incubating them in various salt solutions at different temperatures. Increasing the salt concentration does improve the retention of the DNA at any given temperature (Table 2) but the gain does not appear to be useful, because with increasing salt concentration it is necessary to use higher temperatures for hybridization, and this cancels the advantage of the high salt concentration. For example, the loss in 2 × SSC at 65°C is the same as that in 6 × SSC at 80°C and these are both typical hybridization conditions. Further experiments showed that it is disadvantageous to perform hybridization at high salt concentrations, below the optimum temperature. The optimum temperature for rate of hybridization of *X. laevis* 28 S RNA is around 80°C in 6 × SSC but the rate at 70°C is still appreciable (Fig. 5). Below 70°C the rate falls rapidly. 28 S RNA was hybridized

TABLE 2

Effects of temperature and solvent on retention of sonicated DNA on cellulose nitrate filters

Solvent	50°C	65°C	80°C	90°C
		Temperature DNA retained (%)		
2 × SSC		77	62	48
6 × SSC		97	76	56
10 × SSC		95	83	73
20 × SSC		97	88	81
6 × SSC in 50% formamide	58	50		

^{3}H-labelled *X. laevis* DNA (spec. act. approx. 5×10^5 c.p.m. per μg) was dissolved in ice-cold 0·1 × SSC and sonicated in six 15-s bursts. Between each treatment the solution was cooled in ice for 1 min. The solution was boiled for 5 min, made to 20 × SSC and cooled. Samples of this solution were pipetted on to 13-mm circles of cellulose nitrate, which were then washed in 2 × SSC at room temperature. Approximately 650 c.p.m. were loaded on each filter, and there was no loss caused by washing in 2 × SSC. The filters were dried, baked at 80°C for 2 h in a vacuum oven and immersed in 10 ml of the solvent equilibrated at the temperature used for incubation. After 90 min, the filters were removed, washed in 2 × SSC at room temperature, dried under vacuum and counted in a liquid scintillation counter.

to high molecular weight and sonicated DNA in 6 × SSC at 70 and 80°C (Fig. 6). As expected, the rate of hybridization at 70°C was lower than the rate at 80°C, but against expectation, both the rate and the final extent of hybridization were lower at the lower temperature, for the sonicated but not for the high molecular weight DNA. This result was unexpected because Melli & Bishop did not find an effect of DNA size on the rate of hybridization. They suggested that the decrease in yield for low molecular weight DNA is due to a loss of hybrid from the filter and it would be expected that such losses would increase with temperature. The lower yield for low molecular weight DNA at low temperature remains unexplained, but shows that there is no advantage to be gained in using high salt concentrations and low temperatures to retain small fragments of DNA during hybridization reactions. The advantage of using 6 × SSC at optimum temperature is that the rate is greatly increased over the rate with, say, 2 × SSC. A disadvantage is that the background of RNA that sticks to filters that have no DNA, increases with increasing salt concentration.

(g) *Methods of detecting and measuring hybrids: advantages of film detection*

Radioactive RNA may be detected and measured either by radioautography (or fluorography for weak β-emitters) or by cutting the strip into pieces, which can be counted in a scintillation counter. Film detection methods have the advantages over

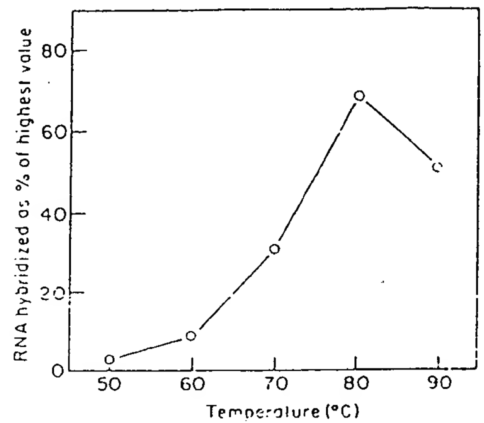

FIG. 5. Temperature dependence of hybridization of 28 S rRNA to *X. laevis* DNA.

X. laevis DNA was loaded on cellulose nitrate filters (17 μg DNA/13-mm diameter disc), which were pretreated as usual for hybridization. ^{3}H-labelled 28 S RNA from *X. laevis* kidney cells (spec. act. $1{\cdot}5 \times 10^6$ c.p.m./μg) was dissolved in $6 \times$ SSC (0·28 μg/ml) and warmed to the temperature used for hybridization. Two filters loaded with DNA and 2 blank filters were introduced into the solutions and left for 30 min. They were washed in 2 l of $2 \times$ SSC at room temperature, treated with 200 ml of RNAase A (20 μg/ml in $2 \times$ SSC) at room temperature for 20 min, washed in 200 ml of $2 \times$ SSC for 10 min, dried under vacuum and counted. Hybridization is expressed as a percentage of that obtained after 5 h at 80°C.

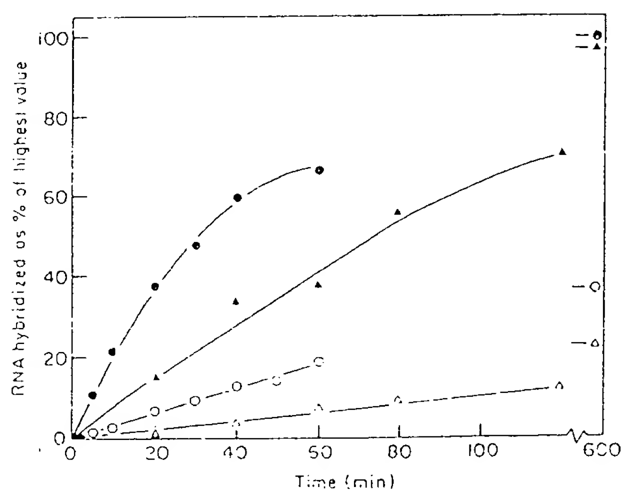

FIG. 6. Time course of hybridization of 28 S RNA to sonicated and high molecular weight DNA at 70 and 80°C.

Filters were loaded as described in the legend to Fig. 5. Two sets were loaded: one with high molecular weight DNA and one with DNA sonicated as described in the legend to Table 2. Hybridization and subsequent treatment of the filters was carried out as described in the legend to Fig. 6 and filters removed at the times indicated. $6 \times$ SSC at 80°C, high molecular weight DNA (●); $6 \times$ SSC at 70°C, high molecular weight DNA (▲): $6 \times$ SSC, 80°C sonicated DNA (○): $6 \times$ SSC at 70°C, sonicated DNA (△).

counting that they are more sensitive, give higher resolution, and can reveal artifacts not seen by counting.

The high sensitivity is illustrated by the analysis of *E. coli* rDNA (Plate I(b)). None of the bands that is clearly visible in the radioautograph contained more than 10 c.p.m. The strip of cellulose nitrate was cut into 150, 1-mm pieces and the pieces counted in a liquid scintillation counter. None of the pieces gave counts more than twice background and none of the features visible in the radioautograph was discernible from the counts. Around 100 c.p.m. of ^{32}P in a single band 1 cm wide can be detected with an overnight exposure. The radioautograph shown in Plate I was exposed for 1 week. Fluorography of ^{3}H is not so sensitive; about 3000 d.p.m. in a 1-cm band are needed to give a visible exposure overnight. The fluorograph shown in Plate II was exposed for 2 months.

The greater resolution of film detection is illustrated by a comparison of Plate II with Figure 7(c). Plate II is a fluorograph of the strip and Figure 7(c) shows the pattern of counts obtained by cutting the strip into 1-mm pieces. Many of the bands seen in the fluorograph are not discernible in the pattern of counts (compare also the tracing of the fluorograph (Fig. 7(b)) with (c)).

For ionizing radiation, blackening of the X-ray film is proportional to the amount of incident radiation, up to the limit where a high proportion of silver grains are exposed. The relative amount of radioactivity in bands can therefore be compared by tracing radioautographs in a densitometer and comparing peak areas. However, like all other photosensitive materials, X-ray films suffer from "reciprocity failure" at low intensities of illumination by non-ionizing radiation and it is likely that bands which contain only a few counts of ^{3}H will not be detected by fluorography even after long exposures. I have not determined the lower limit of detection. Bonner & Laskey (1974) found that 500 d.p.m. of ^{3}H in a band 1 cm × 1 mm could be detected in one week and in my own experience, less than 20 d.p.m. can be detected with longer exposure. Reciprocity failure could affect quantitation of fluorographs by densitometry but comparison of Figure 7(b) and (c) suggests that the response of the film is linear within the limits of this experiment. Clearly, quantitation of ^{32}P by densitometry can be accurate and more sensitive than counting, but film response to ^{3}H may not be linear for low amounts.

An additional advantage of film detection is that non-specific binding of RNA to the cellulose nitrate is more easily distinguished from bands of hybrid. Plate III illustrates this point. In this radioautograph, non-specific binding can be seen as dots and streaks with an appearance clearly different from that of a band. Had this strip been analysed by counting, non-specific binding would not have been distinguishable from the hybrids.

(h) *Analysis of ribosomal DNA in* X. laevis

A total of 0·6 μg of purified *X. laevis* rDNA was digested with EcoRI and the fragments separated by electrophoresis in 1% agarose gels (Plate II(c)). The pattern of fragments is similar to that described by Wellauer *et al.* (1974). They compared the secondary structures of the denatured DNA fragments with those of the ribosomal RNAs and showed that the fastest running fragment (M_r approx. 3×10^6) contained most of the DNA coding for 28 S RNA, all of the transcribed spacer, and a small portion of the DNA coding for 18 S RNA. The larger fragments (M_r 4 to 6×10^6) contained most of the DNA coding for 18 S RNA, all of the non-transcribed

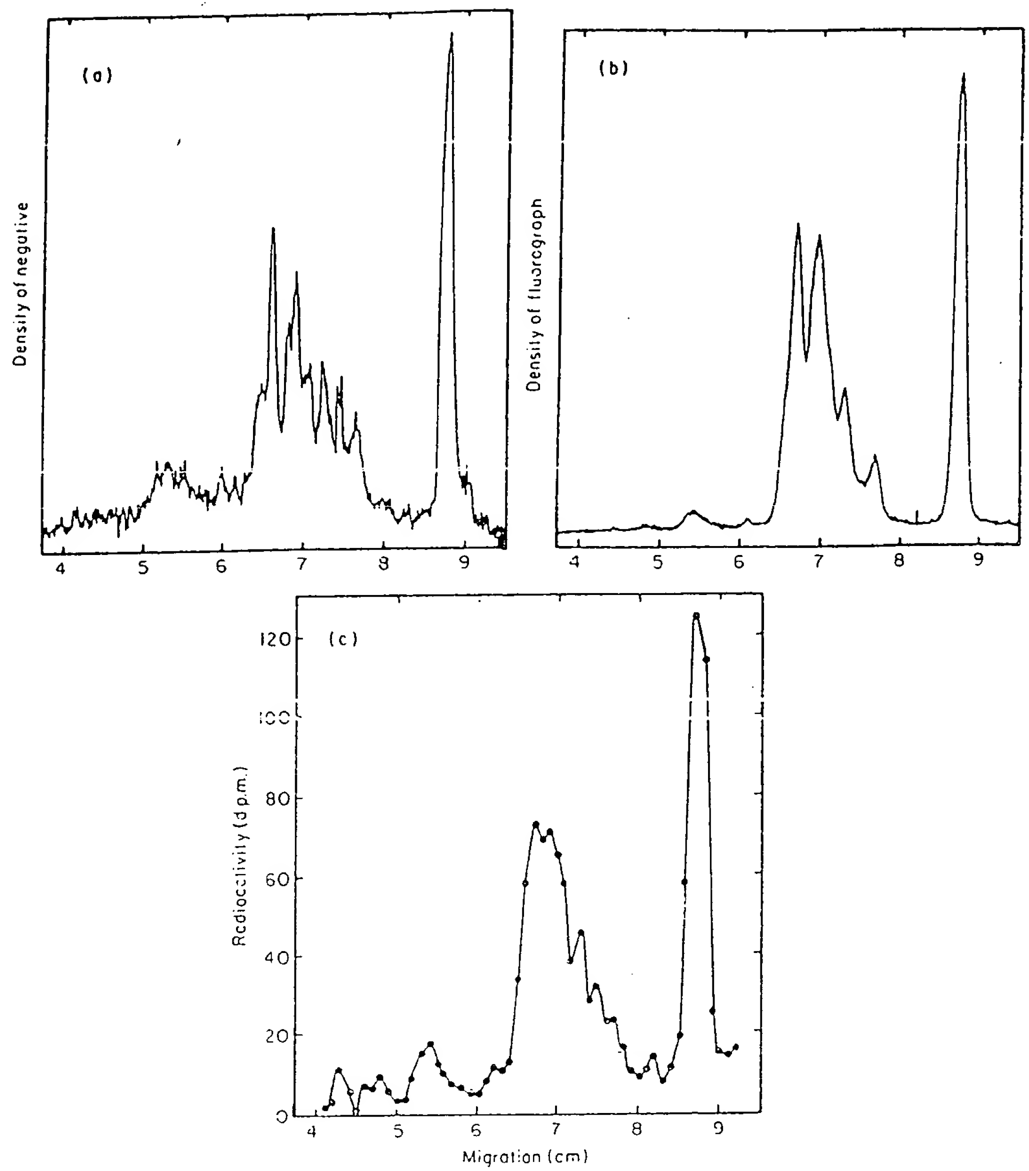

FIG. 7. (a) Microdensitometer tracing of the negative of Plate II(c). (b) Microdensitometer tracing of Plate II(a). (c) Distribution of counts in the Millipore strip which on fluorography gave Plate II(a). The strip was cut into 1 mm pieces, which were counted in a liquid scintillation counter at an efficiency of 40%.

spacer, and a small portion of the DNA coding for 28 S RNA. Different lengths of non-transcribed spacer DNA accounted for the variation in size of the longer fragments. The digest shown in Plate II(c) was transferred to cellulose nitrate as described previously. The strip was cut longitudinally into 2 parts and 1 part was hybridized with 18 S RNA and the other with 28 S RNA. Hybrids were detected by fluorography of the ^{3}H-labelled RNA (Plate II(a) and (b)). Comparison of Plate II(a) and (c)

shows that the resolution of the fine bands containing the 18 S coding sequence is not as high in the fluorograph as it is in the photograph of the gel. Whereas 9 bands can be distinguished in the photograph, only 7 can be distinguished with confidence in the fluorograph. From this analysis it is possible to locate the EcoRI site within the DNA coding for 18 S RNA. As Wellauer *et al.* (1974) showed, 1 of the 2 breaks in the rDNA occurs towards one end of the 18 S region and the other is close to the distal end of the 28 S region. The 3×10^6 mol. wt fragment accounts for virtually all of the hybridization to 28 S RNA and for about 30% of the hybridization to the 18 S RNA (27% measured from the tracing of the fluorograph (Fig. 7(b)) and 31% from the counts). Only traces of 28 S RNA hybridize to the heterogeneous collection of fragments with molecular weights between 4 and 6×10^6, whereas about 70% of the 18 S hybridization is accounted for in these fragments. Thus the break in the 28 S region of the DNA is very close to the end of the coding sequence and the break in the 18 S region is about one-third of the way into the coding sequence.

(i) *Analysis of mouse and rabbit ribosomal DNAs: evidence for long, non-transcribed spacer DNA*

An EcoRI digest of total mouse DNA was separated by electrophoresis on cylindrical 1% agarose gels and transferred to strips of cellulose nitrate paper. One strip was hybridized to 18 S RNA and another to 28 S RNA prepared from rat myoblasts labelled with ^{32}P. The 28 S hybrids showed a strong, sharp band at the position of about $5{\cdot}2\times10^6$ daltons and a very faint, broad band in the region around 14×10^6 daltons (Fig. 8(b)). The 18 S hybrids showed corresponding bands but in this case the slower moving, broad band was relatively more intense (Fig. 8(a)). From this information, a partial structure can be derived for the ribosomal DNA in mouse. Assuming that the ribosomal genes are tandemly linked, it is clear that EcoRI makes at least 2 breaks in the sequence; one in the 18 S and one in the 28 S region. Transcription of ribosomal genes in mammals produces a precursor RNA corresponding to a DNA mol. wt of about 6×10^6, and it follows that the EcoRI fragment of about $5{\cdot}2\times10^6$, which contains both 28 S and 18 S sequences, must also encompass much of the transcribed spacer. The heterogeneous fragments with a mol. wt of 14×10^6 must contain a long stretch of non-transcribed spacer, and may contain some of the transcribed spacer too.

A similar analysis was carried out with rabbit DNA and gave similar results, although the size of the fragments was different from the corresponding fragments from mouse DNA. The band containing most of the 28 S sequence was larger (M_r approx. 6×10^6), whereas that containing most of the 18 S sequence was smaller (M_r approx. 12×10^6) and more homogeneous than the corresponding fragment in the mouse. The structures of mouse and rabbit ribosomal DNAs are thus rather similar to that of *X. laevis* but with longer spacer regions. The overall length of the unit in mouse is at least twice as long as that in *X. laevis*.

(j) *EcoRI sites in the rDNA of five mammals*

The analyses described above, taken with those of Wellauer *et al.* (1974) suggest that the two EcoRI sites in the ribosomal genes have been conserved since the amphibians and mammals diverged. In this case it would be expected that all

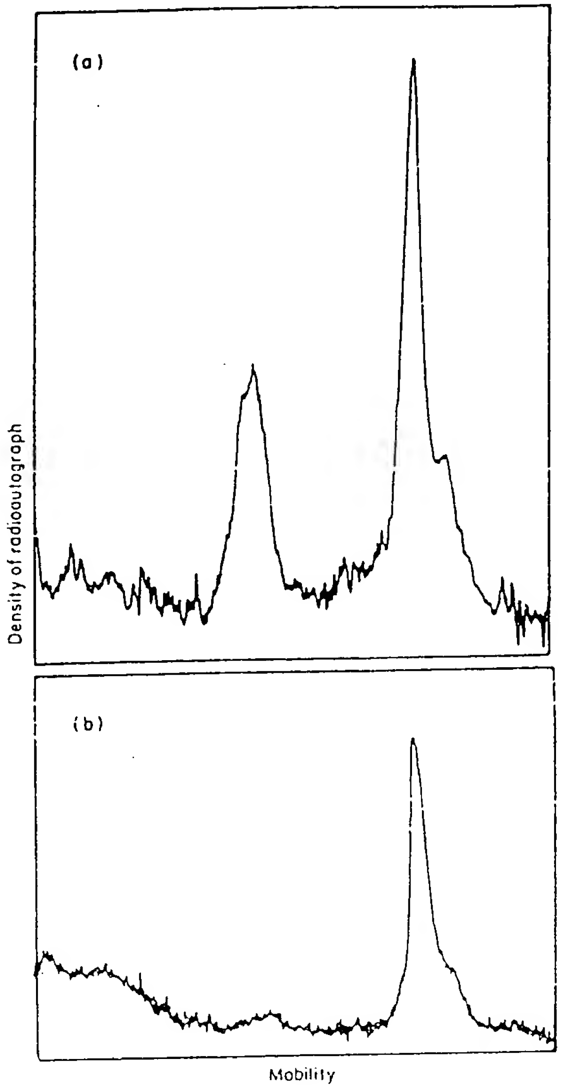

FIG. 8. EcoRI digest of mouse DNA hybridized to 18 S and 28 S RNA.

Total mouse DNA was digested to completion with EcoRI. The digest was separated by electrophoresis on 1% cylindrical agarose gels (9 mm × 24 cm, 5 mA/tube for 20 h, 40 μg of DNA/gel). The gels were stained, photographed, and the DNA transferred to cellulose nitrate as described in Materials and Methods. One gel was hybridized to ^{32}P-labelled 18 S RNA and another to 28 S RNA. The RNA concentration was 0·1 μg/ml in 6 × SSC and hybridization was carried out at 80°C for 4 h. The filters were then washed in 2 × SSC (4 l) at 60°C for 30 min, dried and radioautographed using Kodak Blue Brand X-ray film.

(a) Densitometer tracing of the 18 S hybrids. (b) Densitometer tracing of the 28 S hybrids.

mammalian rDNAs would have equivalent EcoRI sites. Total DNAs from calf thymus, human placenta, and from livers of mouse, rabbit and rat were digested with EcoRI and the fragments separated by electrophoresis on cylindrical gels. The fragments were then transferred to a single sheet of cellulose nitrate filter and hybridized with ^{32}P-labelled rat 28 S RNA. All 5 DNAs showed a strong band in the radioautograph

of the sheet. Each band was in the mol. wt region of 5 to 6×10^6, but there were small differences in their mobilities (Table 3). This result suggests that the two EcoRI sites have indeed been conserved in the rDNA of the mammals. The different fragment size

TABLE 3

Size of EcoRI fragments that hybridize to ribosomal RNAs

Species	Size of RI fragment bearing 28 S sequences ($\times 10^{-6}$)	Size of fragments bearing 18 S sequences ($\times 10^{-6}$)
Calf	5·7	
Human	5·7	
Mouse	5·2	5·2 and approx. 14
Rabbit	6·0	6·0 and approx. 12
Rat	6·0	
X. laevis	3·0	3·0 and 4 to 6

Sizes were estimated from mobilities in 1% agarose gels by comparison with EcoRI fragments of λ-phage DNA. The sizes of the large fragments from mouse and rabbit DNAs hybridizing to 18 S RNA are approximate estimates because there was only one marker in this region of the gel and in this region large differences in size result in small mobility differences.

can readily be accounted for by differences in the size of the transcribed spacer between 28 S and 18 S regions. Different sizes for the ribosomal RNA precursor have been reported for HeLa cells and mouse L-cells (Grierson *et al.*, 1970).

3. Conclusion

The method described here provides a simple way of detecting DNA fragments that are complementary to RNAs, after the DNA frqgments have been separated by gel electrophoresis. Transfer of the DNA from the gel to the cellulose nitrate filter is almost complete for a wide range of fragment sizes. However, large fragments ($M_r > 10^7$) diffuse rather slowly and small fragments hybridize inefficiently. These factors should be taken into account when the method is used for quantitative work.

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Vol. 98
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Medical Sciences

Detection of sickle cell β^S-globin allele by hybridization with synthetic oligonucleotides

(sickle cell anemia/prenatal diagnosis/genetic disease)

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ABSTRACT Two 19-base-long oligonucleotides were synthesized, one complementary to the normal human β-globin gene (β^A) and one complementary to the sickle cell β-globin gene (β^S). The nonadecanucleotides were radioactively labeled and used as probes in DNA hybridization. Under appropriate hybridization conditions, these probes can be used to distinguish the β^A gene from the β^S allele. The DNA from individuals homozygous for the normal β-globin gene ($\beta^A\beta^A$) only hybridized with the β^A specific probe; the DNA from those homozygous for the sickle cell β-globin gene ($\beta^S\beta^S$) only hybridized with the β^S specific probe. The DNA from heterozygous individuals ($\beta^A\beta^S$) hybridized with both probes. This allele-specific hybridization behavior of oligonucleotides provides a general method for diagnosis of any genetic disease which involves a point mutation in the DNA sequence of a single-copy gene.

Synthetic oligodeoxyribonucleotides have been shown to hybridize specifically to complementary DNA sequences (1–3). Under appropriate hybridization conditions, only perfectly base-paired oligonucleotide·DNA duplexes will form; duplexes containing a single mismatched base pair will not be stable. This high degree of hybridization specificity has led to the development of a general method for using synthetic oligonucleotides as specific probes to identify cloned DNAs coding for proteins of interest. Recently, this technique has been applied to the successful isolation of a human β_2-microglobulin cDNA clone (4) as well as a murine transplantation antigen cDNA clone (5).

Because a mutation in a single base in the DNA sequence of a gene would affect the hybridization behavior of an oligonucleotide complementary to the region of the mutation (2), oligonucleotide hybridization has the potential to provide a method of detecting single-base changes within genomic DNA. Point mutations are the cause of a substantial number of human genetic diseases (6). Synthetic oligonucleotides, therefore, could be used as specific probes for determination of genotype and aid in diagnosis of genetic disease, even prenatally.

We chose the β-globin gene as a model system to test the applicability of using synthetic oligonucleotides to detect a point mutation within a single copy gene. The β-globin gene is a member of the single-copy β-globin-like gene family which includes the ε-, ${}^G\gamma$-, ${}^A\gamma$-, δ-, and β-globin genes arranged 5′ to 3′ in order of expression during development (7). Recently, these genes have been cloned, restriction maps have been defined, and much of the DNA sequence has been determined for the coding and immediate flanking regions (8–11). Sickle cell anemia, a human genetic disease found predominantly in the Black population, is the result of a single base pair (bp) change (adenine to thymine) in the β-globin gene, corresponding to the sixth amino acid residue (changing glutamic acid to valine) in the β-globin protein (12, 13). The sickle cell disorders follow a single-gene Mendelian mode of inheritance. Instead of the normal β-globin genotype ($\beta^A\beta^A$), individuals with sickle cell trait have one normal β-globin gene and one sickle cell allele ($\beta^A\beta^S$); those with sickle cell disease have two sickle cell alleles ($\beta^S\beta^S$) and no β^A gene.

In this report, we show that nonadecanucleotides complementary to the β-globin gene (β^A) or to the sickle cell allele (β^S) in the region of the sickle cell point mutation can: (i) distinguish β^A from β^S as well as from other members of the β-globin-like gene family, (ii) specifically detect the single-copy β-globin gene in human genomic DNA, and (iii) allow the unambiguous determination of β-globin genotype of individuals, confirming the hematological diagnosis of sickle cell trait or sickle cell disease. Applied to prenatal diagnosis of sickle cell disorders, this procedure offers several advantages over methods now available. More significantly, these techniques may be applied generally for diagnosis of genetic diseases that involve a specific change—such as a base substitution, insertion, or deletion—in the DNA sequence of a single gene.

MATERIALS AND METHODS

Chemical Synthesis of Oligodeoxyribonucleotides of Unique Sequence. Nonadecanucleotides (Table 1) were synthesized on a solid support by the modified triester approach as described (14).

^{32}P-Labeling of Synthetic Oligonucleotides. Synthetic oligonucleotides were labeled with adenosine 5′-[γ-^{32}P]triphosphates (ICN, crude preparation, >7,000 Ci/mmol; 1 Ci = 3.7 × 10^{10} Bq) by a kinase reaction (1). Separation of labeled oligonucleotide from unlabeled nonadecanucleotide and reaction products by homochromatography (15) yielded oligonucleotide probes with specific activities of approximately 2 × 10^9 cpm/μg.

Source of Recombinant DNA and Transformants. λ-HβG1 and λ-HγG5 and pBR322–HβPst clones were a generous gift of T. Maniatis. The pBR322–HβPst plasmid contained a 4.4-kilobase (kb) *Pst* I fragment of β^A subcloned in the *Pst* I site of pBR322 (16). DNA was isolated as described (3, 17). Recom-

Abbreviations: bp, base pair(s); kb, kilobase(s); β^A, normal β-globin gene; β^S, sickle cell β-globin gene; $\beta^A\beta^A$, normal β-globin genotype; $\beta^A\beta^S$, sickle cell trait genotype; $\beta^S\beta^S$, sickle cell disease genotype; λ-HβG1, recombinant bacteriophage constructed with DNA insert of human δ-globin and β-globin genes; λ-HγG5, recombinant bacteriophage constructed with DNA insert of human ${}^G\gamma$- and ${}^A\gamma$-globin genes; pBR322-HβPst, recombinant plasmid constructed with insert of human β-globin gene.

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Table 1. DNA sequence of synthetic oligonucleotide probes for normal β-globin gene (β^A) and sickle cell β-globin allele (β^S)

Gene	Probe	DNA sequence
β^A	Hβ19A	5'CT CCT GAG GAG AAG TCT GC3'
	Hβ19A'	3'GA GGA CTC CTC TTC AGA CG5'
β^S	Hβ19S	5'CT CCT GTG GAG AAG TCT GC3'
	Hβ19S'	3'GA GGA CAC CTC TTC AGA CG5'

binant DNA was handled in accordance with the National Institutes of Health guidelines.

Isolation of Human DNA. Peripheral blood samples (10–20 ml) were collected from scientific personnel (normal for β-globin) or from patients with sickle cell trait ($\beta^A\beta^S$) or sickle cell disease ($\beta^S\beta^S$); all gave informed consent. DNA was isolated from leukocytes as described (18) with some modifications. Instead of dialysis, the DNA was extracted three times with phenol/chloroform, 3:1 (vol/vol), and treated with RNase (19). The average yield was 50 μg of DNA per ml of whole blood.

Restriction Endonuclease Digestion of DNA. λ-HβG1 DNA and pBR322–HβPst DNA were digested at 1 enzyme unit/μg of DNA at 37°C for 30 min. Human DNA (10 μg) was digested at 5 enzyme units/μg of DNA at 37°C for 4 hr; additional endonuclease was then added at 5 units/μg of DNA and digestion was continued for 12 hr or overnight. The restriction endonucleases used were *Bam*HI, *Eco*RI, and *Hpa* I (Boehringer Mannheim). *Bam*HI and *Hpa* I digestions were carried out in 7 mM NaCl/7 mM Tris·HCl, pH 7.5/7 mM dithiothreitol/7 mM magnesium acetate. The buffer for *Eco*RI was 100 mM Tris, pH 7.2/5 mM magnesium acetate/100 mM NaCl/0.02% Nonidet P-40. When double digestions were required, the amounts of enzyme and times of digestion were as described above, except the endonuclease requiring low-salt buffer was used first. The buffer was then adjusted to higher salt conditions for digestion with *Eco*RI. Digestions were terminated by heating the sample at 65°C for 3 min.

Electrophoresis and Hybridization of DNA. Digested DNAs were loaded into individual wells of a vertical (1 or 3 mm thick) 1.0–1.2% agarose gel (SeaKem), electrophoresed at 1 V/cm at 50 V for 750 V hr in a Tris borate electrophoresis buffer, stained with ethidium bromide (Calbiochem) at 0.25 μg/ml for 30 min, and photographed over UV light (3, 20). The DNA was denatured *in situ* and transferred to nitrocellulose paper (21). Alternatively, the gel itself was dried for direct hybridization (22). After ethidium bromide staining, the gel was denatured with 0.5 M NaOH and 0.15 M NaCl at room temperature for 30 min and neutralized in 0.5 M Tris, pH 8.0/0.15 M NaCl at 4°C for 30 min. The gel was then dried under vacuum onto Whatman 3MM paper with a Hoefer gel dryer. The dry gel was rinsed in 0.15 M NaCl to remove backing paper. Nitrocellulose paper or the dried gel was sealed in plastic (Dazey Seal-A-Meal), hybridized, and washed as specified in the figure legends. The nitrocellulose or gel was blotted dry with Whatman 3MM paper, wrapped in Saran Wrap, and autoradiographed between two Quanta III intensifier screens (DuPont) at −80°C for 4 hr or 3–5 days.

RESULTS

Rationale of Specificity of Oligonucleotide Hybridization. The DNA sequences of the synthetic oligonucleotides used in this study are given in Table 1. The position and length of the sequence of the oligonucleotides were based on several criteria. (*i*) The oligonucleotide was designed to be 19 nucleotides long in order that this sequence would have a high probability of recognizing a unique sequence (23). Hβ19A and Hβ19A' were specific for normal β-globin DNA (β^A); Hβ19S and Hβ19S' were specific for sickle cell β-globin DNA (β^S). (*ii*) The sickle cell mutation was positioned near the center of the sequence to maximize thermal instability of mismatch hybridization. (*iii*) The sequences synthesized were not complementary to the ε-, $^G\gamma$-, $^A\gamma$-, or δ-globin genes in the region of probe complementarity (Table 2).

The Hβ19A' oligonucleotide (or Hβ19A) should form a perfect hybrid with the β^A DNA (Table 3). If the sickle cell point mutation (β^S) is present, there would be one mismatched nucleotide at the site of the point mutation in the oligonucleotide-DNA duplex with Hβ19A' (T/T mismatch) or Hβ19A (A/A mismatch) probe. Conversely, Hβ19S' (or Hβ19S) oligonucleotide probe should hybridize perfectly with β^S DNA. However, one mismatched nucleotide would be present in a duplex of β^A DNA and Hβ19S' (A/A mismatch) or Hβ19S (T/T mismatch) oligonucleotide.

Table 2. Amino acid sequence and DNA sequence of human β-globin-like genes in the region of oligonucleotide hybridization

Gene	5'	1				5					10	3'	Mismatches, no. Hβ19A	Mismatches, no. Hβ19S
ε	...Met	Val	His	Phe	Thr	Ala	Glu	Glu	Lys	Ala	Ala	Val ...		
	...ATG	GTG	CAT	TTT	ACT	GCT	GAG	GAG	AAG	GCT	GCC	GTC...	2	3
$^G\gamma$	...Met	Gly	His	Phe	Thr	Glu	Gly	Asp	Lys	Ala	Thr	Ile ...		
$^A\gamma$	...ATG	GTG	CAT	TTC	ACA	GAG	GAG	GAC	AAG	GCT	ACT	ATC...	7	8
δ	...Met	Val	His	Leu	Thr	Pro	Glu	Glu	Lys	Thr	Ala	Val ...		
	...ATG	GTG	CAT	CTG	ACT	CCT	GAG	GAG	AAG	ACT	GCT	GTC...	1	2
β^A	...Met	Val	His	Leu	Thr	Pro	Glu	Glu	Lys	Ser	Ala	Val ...		
	...ATG	GTG	CAC	CTG	ACT	CCT	GAG	GAG	AAG	TCT	GCC	GTT...	0	1
β^S	...Met	Val	His	Leu	Thr	Pro	Val	Glu	Lys	Ser	Ala	Val ...		
	...ATG	GTG	CAC	CTG	ACT	CCT	GTG	GAG	AAG	TCT	CCC	GTT...	1	0

Italic nucleotides represent position of base-pair differences relative to the β^A. The connecting line represents the position of oligonucleotide hybridization.

Table 3. Type of base-pair mismatch in duplexes of synthetic oligonucleotide probe and DNA of β^A and of β^S

	β-Globin allele	
Probe	β^A	β^S
Hβ19A	Perfect match	A/A mismatch
Hβ19A′	Perfect match	T/T mismatch
Hβ19S	T/T mismatch	Perfect match
Hβ19S′	A/A mismatch	Perfect match

Selectivity of Oligonucleotide Hybridization to Temperature. The effect of temperature on oligonucleotide hybridization to human β-globin DNA is shown in Fig. 1: pBR322–HβPst DNA was digested with *Bam*HI and subjected to electrophoresis in agarose gels in eight identical lanes; the DNA was denatured *in situ* and transferred to nitrocellulose by the standard Southern procedure (21). For each duplicate, one lane was hybridized with 5′-^{32}P-labeled Hβ19A (perfect match) and the other with 5′-^{32}P-labeled Hβ19S (T/T mismatch) (Table 1). The temperature of the hybridization and the washes that followed were then varied as indicated. With hybridization at 45°C and wash at 0°C, hybridization to the 1.8-kb *Bam*HI restriction fragment containing the 5′ end of the β-globin gene was evident with the Hβ19A (perfect match) and, to a lesser extent, with the Hβ19S (T/T mismatch) probe. Based on the length and DNA sequence of the oligonucleotide, it was calculated that 55°C would be a temperature at which the nonadecanucleotide·DNA complex would be stable only if base pairing were perfect (3). Hybridization of the gel at 45°C followed by a wash at 55°C removed hybridization with the Hβ19S probe (T/T mismatch), allowing stable hybridization only with the Hβ19A probe (perfect match). At a hybridization temperature of 55°C, only the Hβ19A (perfect match) probe hybridized to the 1.8-kb restriction fragment. Under these stringent conditions, only perfectly matched oligonucleotide·DNA duplexes were stable. Hybridization at the higher temperature increased specificity but resulted in a slightly decreased signal (compare 45/55 with 55/55 in Fig. 1).

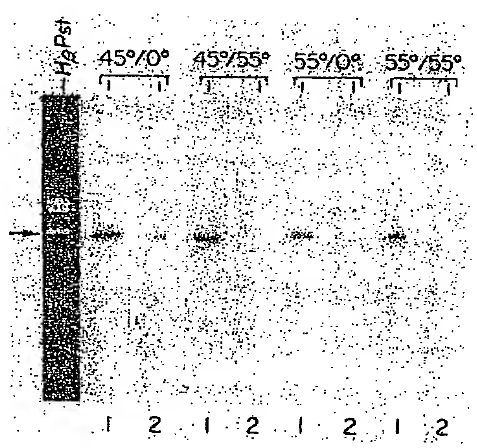

FIG. 1. Effect of temperature on the hybridization of oligonucleotides to globin DNA. Individual lanes of pBR322–HβPst DNA (1 ng) digested with *Bam*HI were hybridized for 16 hr in hybridization buffer [5× Denhardts (1× modified Denhardts is 0.02% bovine serum albumin/0.02% polyvinylpyrrolidone/0.02% $NaDodSO_4$/0.02% Ficoll) containing 10% dextran sulfate (Pharmacia or Sigma), 6× NET (1× NET is 0.15 M NaCl/0.03 M Tris·HCl, pH 8.0/1 mM EDTA), and 0.5% Nonidet P-40]. The buffer also contained (10^6 cpm/ml) of a labeled oligonucleotide probe [5′-^{32}P]Hβ19A (perfect match) (lanes 1) or [5′-^{32}P]Hβ19S (T/T mismatch) (lanes 2). After hybridization at 45°C or 55°C as indicated, each nitrocellulose paper strip was washed with three changes (15 min each) of 0.9 M NaCl/0.09 M sodium citrate at 0°C. Half of the filters were then washed for 1 min at 55°C; as indicated (the numbers at the top of the figure indicate hybridization temperature first and wash temperature second). The ethidium bromide-stained gel is shown at the left. Arrow, position of the 5′ end of the β-globin gene.

Oligonucleotide Probes Can Differentiate β-Globin Gene from Other β-Globin-Like Genes. The stringent hybridization conditions established above were used to determine if the oligonucleotide probes could also distinguish the β-globin gene sequence from those of the β-globin-like genes. The recombinant bacteriophage λ-HβG1 contains both the δ- and β-globin genes; λ-HγG5 contains the human $^G\gamma$- and $^A\gamma$-globin genes (17). There is one base-pair difference in the region of Hβ19A hybridization between δ- and β-globin genes, and seven nucleotide changes in this region between $^G\gamma$- or $^A\gamma$- and β-globin (Table 2). These two DNAs were digested with *Eco*RI and hybridized with 5′-^{32}P-labeled Hβ19A (Fig. 2). At 45°C the labeled probe hybridized to the fragment containing δ-globin (T/T mismatch) as well as to the fragment containing the β-globin gene in λ-HβG1 DNA (perfect match). At 55°C, only hybridization to the fragment containing the β-globin gene was evident. The λ-HγG5 DNA, with seven noncomplementary bases in the region of hybridization, did not hybridize to the probe at either temperature.

Detection of β-Globin Gene in Human Genomic DNA. Because the sequence of the β-globin gene has been determined and extensive restriction maps have been established (11), it is possible to predict the fragment sizes expected to contain the 5′ region of the β-globin gene if total human genomic DNA were digested with various restriction endonucleases. Fig. 3A shows the results for digestion of λ-HβG1 DNA and human genomic DNA normal for the β-globin gene ($\beta^A\beta^A$) with *Bam*HI or double digestion with either *Bam*HI/*Eco*RI or *Hpa* I/*Eco*RI. From the known restriction map of the β-globin gene (11), the sizes of restriction fragments predicted to contain the 5′ end of the β-globin gene are: 1.8 kb, *Bam*HI; 1.8 kb, *Bam*HI/*Eco*RI; 2.2 kb, *Hpa* I/*Eco*RI (Fig. 3B). λ HβG1 was similarly digested and included on the gel as a marker. In the *Bam*HI

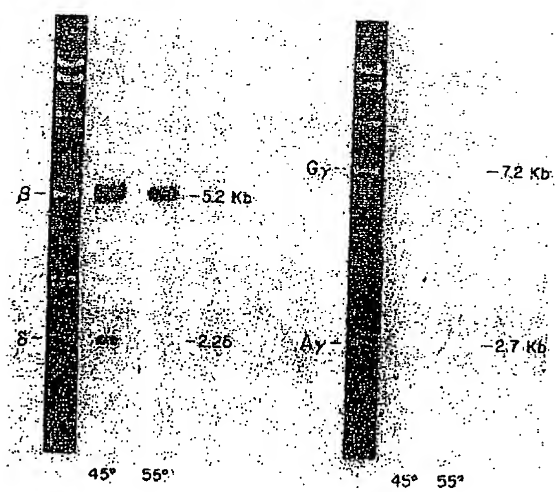

FIG. 2. Hybridization of ^{32}P-labeled Hβ19A to $^G\gamma$-, $^A\gamma$-, δ-, and β-globin genes. Duplicate samples of λ-HβG1 DNA (*Left*) or λ-HγG5 (*Right*) (0.25 μg) were digested with *Eco*RI and hybridized with 5′-^{32}P-labeled Hβ19A probe at either 45°C or 55°C for 18 hr (as in Fig. 1). The positions of the *Eco*RI restriction fragments containing the 5′ end of each globin gene are noted to the left of the ethidium bromide-stained gels.

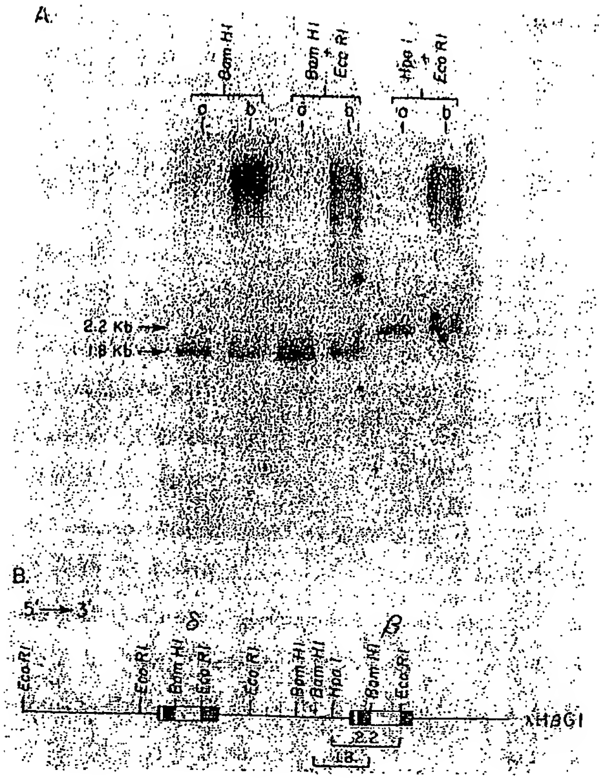

FIG. 3. Hybridization of ^{32}P-labeled Hβ19A′ to human genomic DNA digested with different restriction endonucleases. (A) λ-HβG1 DNA (lanes a) or 10 μg of human genomic DNA (lanes b) ($\beta^A\beta^A$) was subjected to single or double digestion as indicated. After electrophoresis, the agarose gel was dried, hybridized with [5′-^{32}P]Hβ19A′ at 10^7 cpm/ml for 2 hr at 55°C, washed at 0°C (Fig. 1), and then given a 1-min wash in 0.9 M NaCl/0.09 M sodium citrate at 55°C. λ-HβG1 was loaded at 150 pg as a control for intensity of hybridization of a single copy gene, except that in the *Bam*HI/*Eco*RI double digestion 300 pg of DNA was loaded. (B) Localization of selected restriction enzyme sites for *Eco*RI, *Bam*HI, and *Hpa* I (10). The expected sizes of the *Bam*HI or *Bam*HI/*Eco*RI fragment (1.8 kb) and the *Hpa* I/*Eco*RI fragment (2.2 kb) are indicated.

and the *Hpa* I/*Eco*RI lanes, the amount of λ-HβG1 DNA loaded was equivalent to a single-copy gene in 10 μg of human DNA (5×10^{-6} pmol). In each digestion, the hybridizing band in the genomic digests comigrated with the restriction fragments containing the 5′ end of the β-globin gene in the λ-HβG1 marker DNA (Fig. 3A). In addition, the level of hybridization of the hybridizing band in the genomic digests was as expected for a single-copy gene, based on the hybridization obtained for the λ-HβG1 DNA.

Determination of Gene Dosage of β^A and β^A Allele in Human Genomic DNA. Genomic DNAs from patients previously diagnosed as normal for β-globin ($\beta^A\beta^A$) or having sickle cell trait ($\beta^A\beta^S$) or sickle cell disease ($\beta^S\beta^S$) were digested with *Bam*HI endonuclease and subjected to electrophoresis in duplicate. For one half of the gel, the genomic DNAs with an appropriate marker for the 5′-end of β^A gene (λ-HβG1 digested with *Bam*HI) were hybridized with Hβ19A′ probe (β^A, perfect match; β^S, T/T mismatch) (Fig. 4A). The duplicate lanes were hybridized with Hβ19S probe (β^A, T/T mismatch; β^S perfect match) (Fig. 4B). In Fig. 4A, the 1.8-kb *Bam*HI restriction fragment hybridized with Hβ19A′ for normal ($\beta^A\beta^A$) and sickle cell trait ($\beta^A\beta^S$) genomic DNA. The level of hybridization to the 5′ end of the globin gene fragment for normal DNA was noticeably greater than that for sickle cell trait DNA. Very little hybridization was seen with ($\beta^S\beta^S$) DNA with the Hβ19A′ probe (T/T mismatch). When the Hβ19S probe was hybridized in the duplicate lanes, the probe hybridized to the 1.8-kb fragment in the $\beta^S\beta^S$ and $\beta^A\beta^S$ DNAs but not to the $\beta^A\beta^A$ DNA (T/T mismatch) (Fig. 4B). Again, the level of hybridization with the Hβ19S probe was greater with $\beta^S\beta^S$ DNA than with $\beta^A\beta^S$ DNA.

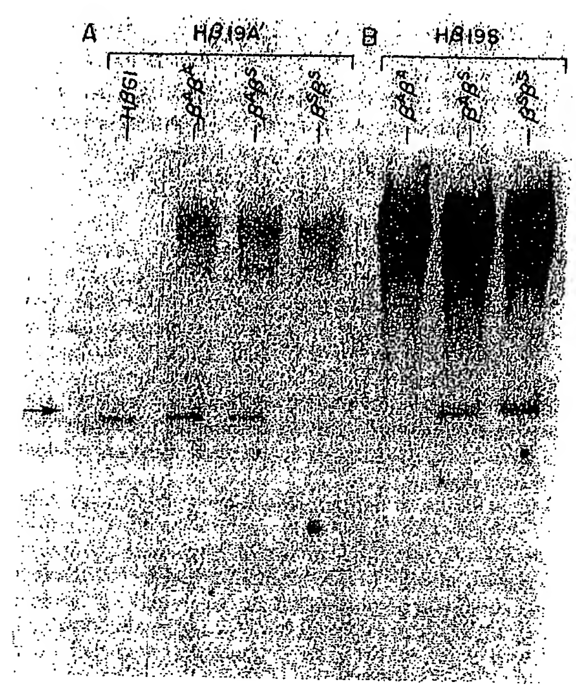

FIG. 4. Hybridization of [^{32}P]nonadecanucleotide (Hβ19A′ and Hβ19S) to human genomic DNA. λ-HβG1 DNA (150 pg) and duplicates of genomic DNAs (10 μg) $\beta^A\beta^A$, $\beta^A\beta^S$, and $\beta^S\beta^S$, were digested with *Bam*HI and electrophoresed. The gel was dried and half was hybridized with [5′-^{32}P]Hβ19A′ (A); the other half was hybridized with [5′-^{32}P]Hβ19S (B) at 10^7 cpm/ml for 2 hr at 55°C. The gels were washed at 0°C and twice, for 1 min each, in 0.9 M NaCl/0.9 M sodium citrate at 55°C. The gel pieces were realigned and autoradiographed. The 1.8-kb fragment containing the 5′ end of the β-globin gene is indicated by the arrow.

DISCUSSION

We have demonstrated in this report that synthetic oligonucleotides recognizing a specific sequence of DNA can detect a single-copy gene in human genomic DNA. The nonadecanucleotide probes can differentiate the β-globin gene from other members of the β-globin-like gene family and can distinguish the normal β-globin gene (β^A) from the β^S allele, a difference of a single nucleotide change.

In this study, 1 bp mismatch out of 19 bp decreased the thermal stability of the oligonucleotide·DNA duplex. Hybridization and wash conditions were established that would allow discrimination of β^A DNA from DNAs containing a single base change such as δ-globin and β^S genes, as well as other genes in the β-globin-like gene family. The type of nucleotide mismatch—e.g., A/A, T/T, etc.—could be expected to have an effect on the stability of the complex. However, our results showed that the hybridization patterns of nonadecanucleotide·DNA duplexes with a T/T bp mismatch [Hβ19A′ with β^S DNA, Hβ19S

with β^A DNA (Fig. 4)] were similar to those obtained with an A/A bp mismatch (Hβ19A with β^S DNA, Hβ19S′ with β^A DNA; data not shown).

The ability of synthetic oligonucleotides to detect a point mutation within this sequence was tested by hybridization with genomic DNA from individuals normal for β-globin ($\beta^A\beta^A$) or having the sickle cell allele (β^S) in the heterozygous or homozygous state. The oligonucleotide probes Hβ19A′ and Hβ19S hybridized to their respective genes in an allele-specific manner (Fig. 4). Significantly, the intensity of labeling at the hybridized β-globin gene fragment for each probe was proportional to gene dosage. The genotype of the β-globin gene could be determined from the hybridization pattern, confirming diagnoses made previously by hemoglobin typing (S. Rahbar, personal communication) (24). The reason for the hybridization in the upper region of the gel for genomic DNA samples (Figs. 3 and 4) is not known. However, it is not likely that this binding represents partial or incompletely digested fragments because of the experimental conditions used.

The technique of hybridization with synthetic oligonucleotides yields results equivalent to analysis with *Mst* II endonuclease (25–27). Both procedures offer direct analysis, and both utilize small amounts of DNA. A limitation of the *Mst* II analysis is that *Mst* II will not distinguish the β^C from the β^S allele because the β^C mutation (GAG to AAG, glycine to lysine, sixth codon, β-globin) occurs at the N position (N = any) of the *Mst* II recognition sequence (C-C-T-N-A-G-G). Oligonucleotide probes, on the other hand, are specific for the β^A and β^S alleles.

The most significant advantage this technique offers is that it has the potential to be applied to the diagnosis of any genetic disease in which a specific change in DNA sequence is involved, particularly in the case of base substitution but also for insertion or deletion not analyzable by any other methods. The sequence of the oligonucleotide can be designed precisely according to need. This eliminates dependence on restriction enzyme recognition site alteration, which has a low probability of occurrence for any given point mutation (23). Therefore, this technique can be applied not only to the diagnosis of the sickle cell allele (β^S) but also to detection of the β^C mutation and the single base changes recently reported for β-thalassemia (see ref. 28 for review), α-thalassemia (29), and α_1-antitrypsin deficiency disorders (30). These point mutations, which do not affect any known restriction endonuclease recognition site, could be detected readily by an appropriate oligonucleotide probe as described herein.

We thank T. Maniatis for the β-globin clones, S. Rahbar for the gift of blood samples, and C. C. Impraim for assistance in the final stages of the work. R.B.W. and K.I. are members of the Cancer Research Center (CA16434) at the City of Hope Research Institute. The stay of C.M. was made possible in part by a grant from the North Atlantic Treaty Organization. This work was supported by National Institutes of Health Grant HL 29516 (R.B.W.) and Health and Welfare Agency, Genetic Disease Section, State of California Grant 81-77521-A1 (B.J.C. and R.L.T.).

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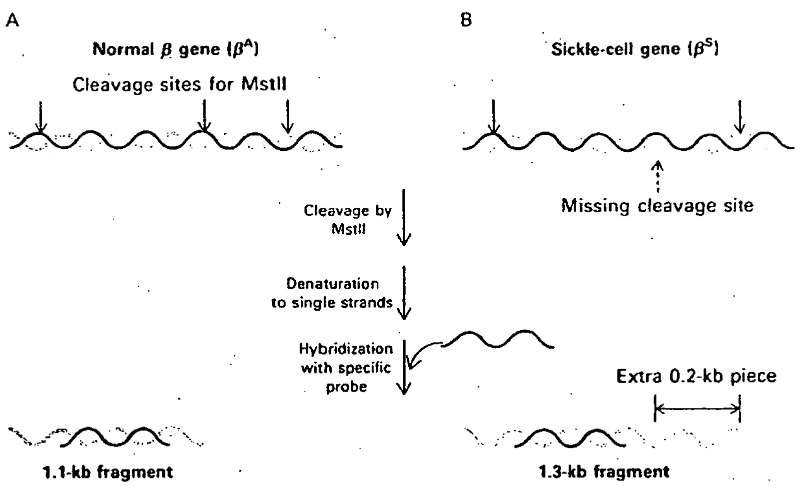
FETAL DNA CAN BE ANALYZED FOR THE PRESENCE OF THE SICKLE-CELL GENE

The substitution of valine for glutamate at $\beta 6$ in hemoglobin S results from a change in a single base, T for A. This mutation can readily be detected by cleaving DNA with a restriction enzyme that recognizes the sequence in this immediate region. The target for the restriction endonuclease MstII is the palindromic sequence CCTNAGG (where N denotes any base), which is present in the gene for the β chain of hemoglobin A (β^A gene) but not in the one for hemoglobin S (β^S). Because of the absence of this target site in the β^S gene, complete digestion of the gene by MstII produces a 1.3-kb fragment, corresponding to a 1.1-kb fragment from the β^A gene (Figure 7-53A). The fragments in the digested sample of DNA are separated by gel electrophoresis and visualized by Southern blotting (p. 120) with a ^{32}P-labeled DNA probe that is complementary to the 1.1-kb fragment. The 1.3-kb fragment is also stained by this probe because it contains the 1.1-kb sequence. An autoradiogram reveals whether the β^A gene, the β^S gene, or both are present in the DNA sample (Figure 7-53B).



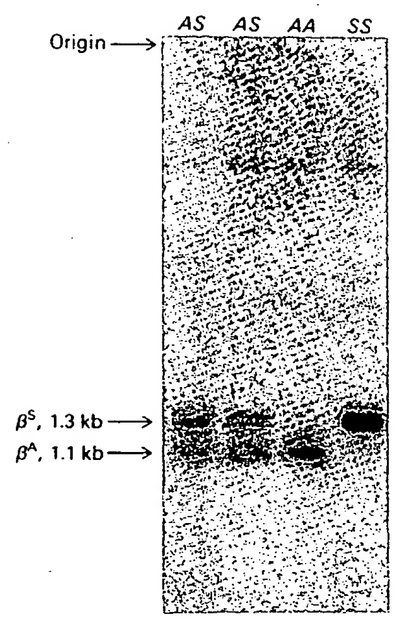


Figure 7-53
Restriction endonuclease method for detecting the sickle-cell gene. (A) Target site in the gene and fragments produced by digestion. (B) Electrophoresis pattern of a digest from parents who are heterozygous for the gene (lanes labeled *AS*), a normal child (*AA*), and a child with sickle-cell anemia (*SS*). [Part B is from Y. W. Kan. In *Medicine, Science, and Society*, K. J. Isselbacher, ed., (Wiley, 1984), p. 297.]

An attractive feature of this restriction enzyme method is that the DNA sample can come from any fetal cell. In contrast, a hemoglobin sample could be obtained only from red blood cells or their precursors. A sample of amniotic fluid is obtained from the fetus more readily and with less potential hazard than are blood-forming cells. DNA analyses can also be performed on biopsy samples of the chorionic villi obtained early in pregnancy, at about eight weeks gestation. These techniques for obtaining and analyzing genomic DNA are generally applicable. The number of genetic diseases that can be detected early in pregnancy by restriction enzyme cleavage of fetal DNA followed by Southern blot-

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Zeta-Probe® Blotting Membranes

Instruction Manual

BIO-RAD

Bio-Rad Laboratories, 2000 Alfred Nobel Drive, Hercules, CA 94547

LIT234 Rev C

Table of Contents

Section 1
Introduction

Zeta-Probe blotting membranes are nylon membranes which have unique binding and handling properties that make them ideally suited for nucleic acid, and some protein, blotting applications.

Zeta-Probe membranes possess a high tensile strength. They won't shrink, tear, or become brittle during transfer, baking, hybridization, or reprobing. Zeta-Probe membranes are heat-resistant, nonflammable, and autoclavable. Zeta-Probe membranes are naturally hydrophilic with no added wetting agents. These membranes are resistant to a wide variety of chemicals, including 100% formamide, 2 M NaOH, 4 M HCl, acetone, most alcohols, DMSO, DMF, and chlorinated aliphatic hydrocarbons. The nominal porosity of Zeta-Probe membranes is 0.45 µm. When stored at 23–25°C, Zeta-Probe membranes are stable for at least 1 year.

When handling Zeta-Probe membranes, always wear gloves or use forceps. After blotting, do not allow wet membranes to come in contact with each other. Contact may result in the transfer of blotted nucleic acids or proteins from one membrane to the other.

Stock buffers are listed in the appendix. It is suggested that you read the entire protocol before proceeding.

Section 2
Nucleic Acid Blotting Protocols

Several nucleic acid blotting methods are presented in this section. Capillary blotting (Sections 2.1through 2.3) is generally used with agarose gels, and electrophoretic transfer (Section 2.4) is used with polyacrylamide gels. Dot blotting (Sections 2.5 and 2.6) is used for

nucleic acids in solution. DNA alkaline blotting (Section 2.3) is an alternative to Southern blotting. DNA alkaline blotting results in higher resolution and greater sensitivity in many applications. DNA alkaline fixation (Section 2.7) can be used to denature and covalently fix DNA to Zeta-Probe membranes after transfer.

2.1 Southern Blotting[1,2] (DNA Capillary Transfer)

1. Depurinate the DNA by soaking the gel in 0.25 M HCl for 10–15 min (be sure that the gel is floating free in all baths).
   Note: Acid depurination is only recommended for fragments >4 kb.
2. Denature the DNA by placing the gel in a bath of 0.5 N NaOH, 1 M NaCl. Place the container on a moving platform for 30 min at room temperature.
3. Neutralize the gel by bathing it in 1.5 M Tris-HCl, pH 7.4, 1.5 M NaCl for 30 min at room temperature on a moving platform. Prepare a Whatman 3MM paper wick. Hang two sheets, prewetted with 10x SSC hung over the sides and into the bottom of the capillary transfer apparatus containing 800 ml 10x SSC. On top of the wick, place two additional sheets of 3MM paper cut to the size of the gel prewetted with 10x SSC.
4. Invert the gel and place it on the wick. Roll a 10 ml plastic pipet, over the gel to remove any bubbles. Trim off the wells from the gel using a spatula.
5. Place membranes labeled side against the gel above the lanes to be transferred. Trim edges of the gel as required with a spatula, or cover exposed areas with Parafilm. Roll with the pipet to remove any air bubbles. It is important to remove air bubbles from underneath the blotting membrane as they will block transfer. To avoid trapping bubbles, place the Zeta-Probe membrane onto the gel surface by first bowing the membrane diagonally and aligning the opposite corners with the gel corners. Then lower the Zeta-Probe membrane onto the gel.

6. Cut two pieces of 3MM paper to the size of the gel. Place both sheets on top of gel. Wet paper with small amount of transfer buffer. Roll with the pipet to remove air bubbles.
7. Flood the surface of the gel with buffer. Carefully place paper towels over the Whatman paper. Stack the paper towels about 15 cm high.
8. Cover the paper towel stack with a glass or plastic plate. Keep the pressure on the paper towel stack at a minimum. Excessive weight will compress the gel, retarding capillary transfer.
9. Keep an excess of buffer in the dish, but do not cover the top of the sponge. Continue transferring for 2–24 hr, depending on the gel concentration and fragment size.
10. After transfer, separate the membrane from the gel, rinse the membrane briefly in 2x SSC, and briefly blot the membrane with filter paper. The DNA can then be fixed onto the Zeta-Probe membrane by baking it at 80°C for 30 min in a vacuum oven. Alternatively, the DNA can be UV-crosslinked to the membrane using 5,000 µJ/cm^2 radiation. Higher levels, although they increase the absolute retention of the nucleic acid on the membrane, can lead to a reduction in signal intensity. The membranes can be stored dry between two pieces of filter paper in plastic bags at 23–25°C.

2.2 Northern Blotting (RNA Capillary Transfer)

Follow the Southern blotting protocol (Section 2.1), omitting steps 1–3. No pretreatment of RNA gels is necessary.[5]

If gels contain glyoxal, remove glyoxal adducts by vacuum baking Zeta-Probe membrane for 1 hour at 80°C after transfer. Alternatively,

pour 95°C 20 mM Tris-HCl, pH 8.0, 1 mM EDTA onto the blotted membrane, then gently agitate at room temperature until the solution cools. After removal of glyoxal adducts, proceed to hybridization or store the membranes dry.

2.3 Alkaline Blotting[3] (DNA Capillary Transfer)

1. Depurinate the DNA by soaking the gel in 0.25 M HCl for 10–15 min. Rinse the gel several times with distilled water.

   Note: Acid depurination is only recommended for fragments >4 kb.
2. Cut four sheets of Whatman 3MM paper so they overhang the bottom of the gel tray by 5 cm on each end. Prewet the Zeta-Probe membrane in distilled water.
3. Place the four sheets of 3MM paper on an inverted gel casting tray. Place the 3MM/tray in the bottom of a deep dish. Then saturate the 3MM paper with 0.4 M NaOH. Remove the bubbles by repeatedly rolling a glass pipet over the saturated 3MM paper. Pour enough NaOH into the deep dish so that the 3MM wick ends are immersed in NaOH.
4. Pour more NaOH onto the 3MM wick to saturate it, then carefully place the gel on the wick. Make sure that no bubbles are trapped beneath the gel. Cover the gel with a small amount of NaOH.
5. Place plastic wrap (such as Saran wrap) over the entire gel/3MM stack. Cut out a window with a clean razor blade, allowing only the gel to be exposed.
6. Lower the sheet of pre-wetted Zeta-Probe membrane onto the gel surface, making contact first in the center, then allowing the edges to gradually fold down. Carefully flood the filter surface with NaOH. Make sure that no bubbles are present between the gel and the Zeta-Probe membrane.

7. Cut two pieces of 3MM exactly to the gel size. Wet a sheet of precut 3MM paper in water and place it on the Zeta-Probe membrane/gel stack, then repeat with the second sheet. Remove any bubbles from beneath each layer of 3MM paper.
8. Place a stack of precut paper towels on the 3MM/Zeta-Probe membrane/gel stack. Cover the paper towel stack with a plastic or glass plate. Keep the pressure on the paper towel stack at a minimum. Excessive weight will compress the gel, retarding capillary transfer.
9. Continue transferring for 2–24 hours, depending on the gel concentration and fragment size. Note: Higher background may appear if transfer is longer than 24 hr.
10. After transfer, remove the stack of paper towels. Gently peel the Zeta-Probe membrane from the surface of the gel, rinse it in 2x SSC, and air dry. DNA is fixed to the membrane during transfer, eliminating the need for subsequent fixation. The dried membranes are stable at room temperature. The membranes can be stored dry between two pieces of filter paper in plastic bags at 23–25°C.

2.4 Electrophoretic Transfer

The following protocol was developed for maximum efficiency of electrophoretic transfer. It affords the greatest mobility of DNA and RNA, and the most complete transfer from gel to membrane without excessive heat generation. The buffer (ionic strength and pH) and field strength have been optimized for electrophoretic blotting of DNA and RNA from both agarose and acrylamide gels. For electrophoretic transfer from agarose gels, a heat exchanger must be used, because increased temperatures could melt the agarose gel. The protocol was developed using the Trans-Blot® electrophoretic transfer system with a heat exchanger.

1. Prepare the stock electrophoretic transfer buffer, 20x TAE or 5x TBE.
2. Prepare gels for transfer immediately after electrophoresis:
   A. Electrophoresis Under Denaturing Conditions

      If gel electrophoresis was done under denaturing conditions (*e.g.*, agarose/formaldehyde gels), equilibrate the gel in 0.5x transfer buffer for 10–15 min prior to electrophoretic transfer.
   B. Electrophoresis Under Nondenaturing Conditions
      1. Soak the gel in 0.2 N NaOH, 0.5 M NaCl for 30 min. For polyacrylamide gels, be sure not to exceed 30 min, since limited gel hydrolysis may occur with subsequent swelling during transfer.

         Note: Zeta-Probe membranes will bind nondenatured nucleic acids. Therefore, denaturing is not mandatory before transferring. Yet, after transferring, the blotted Zeta-Probe membrane must be treated with NaOH. Refer to the DNA alkaline fixation procedure (Section 2.7).
      2. After base treatment, neutralize the gel by washing in 5x transfer buffer two times, 10 min each. Then wash the gel once in 0.5x transfer buffer for 10 min.
      3. While gels are being equilibrated, soak the Zeta-Probe membrane at least 10 min in 0.5x transfer buffer.
      4. Fill the electrophoretic transfer cell to half full with 0.5x transfer buffer, and circulate 4°C coolant through the heat exchanger. If possible, place the cell on a magnetic stirring plate and add a stirbar. Circulate buffer in the cell by stirring to maintain uniform temperature during the run.
      5. Prepare the transfer assembly.

Soak one fiber pad by squeezing it while it is submerged in 0.5x transfer buffer. Lay the soaked pad on the open gel holder. Soak a piece of thick filter paper (*e.g.*, slab gel dryer type paper cut to the size of the fiber pad) in the transfer buffer and place it on the fiber pad. Place the gel on the filter paper. Hold the presoaked Zeta-Probe membrane with both hands so that the middle of the membrane is sagging or bowed downward. Allow the middle of the membrane to contact the gel first. Gradually lower the ends of the membrane onto the gel. This process will expel most bubbles from between the gel and the membrane. If there are any remaining bubbles between the gel and membrane, remove them by sliding a test tube or extended gloved finger across the surface.

Note: Maintaining uniform physical contact between the gel and membrane is of critical importance in electrophoretic transfer.

Place a presoaked piece of thick filter paper on the membrane followed by a presoaked fiber pad. Close the gel holder and place it in the transfer cell so that the membrane is on the anode side of the gel (red pole). Add more 0.5x transfer buffer, if necessary, to bring the buffer level to 1 cm below the electrode post.

6. Transfer at 80 V for 4 hours.

   Note: For comprehensive electrophoretic transfer instructions, including protocols, technical discussion, and troubleshooting guide, refer to the Trans-Blot cell operating manual.
7. After transfer, separate the membrane from the gel, rinse the membrane briefly in 1x transfer buffer, and briefly blot

the membrane with filter paper. Fix nucleic acids onto the Zeta-Probe membrane by baking it at 80°C for 30 min. The membranes can be stored dry between two pieces of filter paper in plastic bags at 23–25°C.

2.5 DNA Dot Blotting

When Zeta-Probe membrane is used, it is not necessary to extract DNA from tissue samples for dot blot analysis. Regardless of whether the sample is purified DNA (covalently closed circular DNA, double-stranded DNA, single-stranded DNA), whole blood, tissue, or cultured cells, it can be heated in alkali, then filtered directly onto the Zeta-Probe membrane.

1. Heat the sample in a total volume of 0.5 ml with a final concentration equal to 0.4 M NaOH, 10 mM EDTA at 100°C for 10 min.[16] The sample may be purified or crude DNA (≤5 µg), whole soft tissue, e.g., liver (≤0.5 mg), whole blood (≤10 µl), cultured cells (≤10^5 cells).
2. Wet a sheet of Zeta-Probe membrane by immersing it in distilled water.
3. Assemble the microfiltration apparatus with the prewetted Zeta-Probe membrane. Make sure that all screws and clamps have been tightened under vacuum to prevent contamination between wells. Rinse wells with 0.5 ml TE or H_2O. Apply vacuum until wells are empty but not dry.
4. Apply a 0.5 ml DNA sample into each appropriate well without vacuum.
5. Start vacuum until the wells are just dry.
6. Rinse all wells by placing 0.5 ml of 0.4 M NaOH in each, then apply vacuum until all wells are quite dry.

7. Disconnect the vacuum, disassemble the apparatus, and rinse the membrane briefly in 2x SSC. UV-crosslink the DNA to the membrane or vacuum dry the blotted Zeta-Probe membrane at 80°C for 30 min. The membranes can be stored dry between two pieces of filter paper in plastic bags at 23–25°C.

2.6 RNA Dot/Slot Blotting

Both native and denatured RNA are retained quantitatively by Zeta-Probe membrane. However, to insure optimal hybridization, RNA samples must be totally denatured before fixing onto the Zeta-Probe membrane.

Glyoxal RNA Denaturation and Fixation

1. Add RNA sample to the following final concentrations:
   50% dimethyl sulfoxide (DMSO)
   10 mM sodium phosphate, pH 7
   1 M glyoxal
2. Incubate sample for 1 hr at 50°C. Then cool the RNA sample on ice.
3. Wet a sheet of Zeta-Probe membrane by immersing it in distilled water.
4. Assemble the microfiltration apparatus with the prewetted Zeta-Probe membrane. Make sure that all the screws or clamps have been tightened under vacuum to prevent cross well contamination.
5. Place a 0.5 ml RNA sample into each appropriate well without vacuum.
6. Apply vacuum until the wells are just dry, then release vacuum.
7. Rinse all wells with 0.5 ml TE, and apply vacuum until the wells are completely dry.

8. Disconnect the vacuum. Remove the blotted Zeta-Probe membrane.
9. Remove the glyoxal by rinsing the membrane in 2x SSC and letting it airdry. Fix RNA onto Zeta-Probe membrane by baking the membrane at 80°C for 1 hr.

2.7 DNA Alkaline Fixation

After transfer, place the Zeta-Probe membrane (DNA surface facing up) on a pad of 3MM paper saturated with 0.4 M NaOH for 10 min. Rinse in 2x SSC and air dry. The dried membranes are stable at room temperature. The membranes can be stored between two pieces of filter paper in plastic bags at 23–25°C.

Section 3
Probe Recommendations

The specific activity, concentration, size range, and purity of the probe all have an important effect on signal-to-noise ratio during hybridization. For hybridization on Zeta-Probe blotting membranes, the following is recommended:

Probe specific activity	10^8 cpm/µg probe
Probe concentration in the hybridization mixture	10^6 cpm/ml (10–50 ng/ml)
Probe length	200–1,000 bp

Probe length is an important parameter to control. DNA probes prepared by random priming tend to be small. Small probes can cause lane specific background during low stringency hybridizations. DNA probes prepared by nick translation are generally long. Probe fragments longer than 1 kb decrease hybridization specificity.

Alternative hybridization protocols are necessary when probe lengths vary outside this recommended range (refer to Oligonucleotide Protocol, Section 4.4).

Template purity is essential during probe synthesis, especially probes made by random primer extension. Small amounts of contaminating DNA templates can cause lane background or extra bands due to the high specific activity of random priming.

Optimal probe specific activity and concentration can vary according to available hybridization sites and exposure time. Probe cleanup is essential to minimize background. Unincorporated nucleotides present after probe preparation contribute to hybridization background. The most effective cleanup method is desalting by column separation. This can be done in a column (1 to 5 ml bed volume) using Bio-Gel® P-30 gel (catalog #150-1340) or with Bio-Spin® 30 columns (catalog #732-6004).

After cleanup, denature the double-stranded probe by increasing temperature to 95–100°C for 5 min. Then cool rapidly on ice. Use the probe as soon as possible after preparation.

Section 4
Hybridization Protocols for DNA Probes

There are several hybridization protocols that will give high quality results. The key to successful nucleic acid blotting is proper blocking of the Zeta-Probe membrane. The most important blocking reagent in the hybridization solution is sodium dodecylsulfate (SDS). SDS is most effective when used at concentrations ^{3}1% (w/v). The Standard Protocol (Section 4.1) uses 7% (w/v) SDS, which has been shown to give extremely low background and high signals. The protocol described in Section 4.2 includes formamide, which allows

hybridization to be performed at a lower temperature. The protocol in Section 4.4 is recommended for oligonucleotide probes. The Alternative Protocol (Section 4.3) should be used only when extreme sensitivity is needed.

The final volume of hybridization solution is important in reducing background. For prehybridization, use 150 µl solution/cm² Zeta-Probe membrane. For washes, use at least 350 µl solution/cm² Zeta-Probe membrane.

One of the most significant advantages offered by Zeta-Probe membrane over conventional membranes is that target nucleic acids of all sizes can be fixed irreversibly. The stringency of hybridization can therefore be optimized for detection of specific target sequences. There is no need to use high ionic strength and low temperature to minimize the loss of nucleic acids from the membrane during hybridization or washing procedures.

Hybridizations should be conducted at 20–25°C below the melting temperature (T_m) of the probe duplex to insure optimal rates of specific hybridization while minimizing interaction with partially homologous sequences.[10] The stringency of post-hybridization washes is less critical, but a good rule of thumb is to conduct the most stringent wash at 10–15°C below T_m.[11] The protocols described below are suitable for probes having a (G+C) content representative of the mammalian genome, i.e., 0.42. If desired, conditions can be varied in accordance with the following empirical formula:

T_m (DNA/DNA) = 81.5 + 16.6 x log [Na] – 0.65 x (% formamide) + 41 x (G + C).11

T_m (RNA/RNA) = 79.8 + 18.5 x log [Na+] – 0.35 x (% formamide) + 58.4 x (G + C) + 11.8 x (G + C)2 12

T_m (DNA/RNA) = approx. mean of T_m (DNA/DNA) and T_m (RNA/RNA)

The T_m is decreased approximately 1.5°C for every 1% decrease in homology.[10, 11]

The T_m is decreased as the fragment length of the probe decreases; the appropriate correction factor is approximately –500 / (# bp in probe fragment)°C.[10, 11]

The rate of hybridization increases as the salt concentration increases.[10]

The rate of hybridization decreases as the formamide concentration increases.[10, 13]

The hybridization temperature (TH) appropriate to synthetic oligomeric DNA probes in 1 M Na+ can be approximated by the following:

T_H + 2 x (no. of A-T bp) + 4 x (no. of G-C bp) –5.14

4.1 Standard Protocol

Prehybridization

1. Seal the blotted Zeta-Probe membrane inside a heat-sealable plastic bag.
2. Cut one corner of the plastic bag and pipet prehybridization solution in:

   0.5 M Na_2HPO_4, pH 7.2
   7% (w/v) SDS
3. Incubate briefly at 65°C for 5 min. The goal is to evenly and completely coat the membrane with this solution.

Hybridization

1. Cut one corner of the plastic bag, remove the prehybridization solution, and replace it with the same buffer.

2. Add the denatured probe and remove all bubbles before resealing the bag. Hybridize for 4–24 hours at 65°C with agitation.
3. Carefully remove the hybridization solution by cutting one corner. Remove hybridized Zeta-Probe membrane plastic bag.

   Note: At no stage before washing should the membranes be permitted to dry.

Washes

1. Wash the membrane at 68°C, 2 times for 10 min each, in the following:

   1x SSC

   0.1% (w/v) SDS

   The first wash should be conducted at room temperature; the second wash should be conducted in the hybridization oven.
2. Wash the membrane at 65°C, 2 times for 30–60 min each, in the following:

   0.1x SSC

   0.1% (w/v) SDS

   These washes should be conducted in the hybridization oven.
3. After washing, the blotted membranes are ready for autoradiography. If no further cycles of hybridization are to be done on the membrane, the membrane can be dried. When reprobing, do not allow the membrane to dry between hybridizations. Expose moist membranes between plastic wrap or enclosed in a sealable plastic bag. Do not allow a wet membrane to come in contact with the film, because wet Zeta-Probe membrane will stick to the film.

4.2 Formamide Protocol

Prehybridization

1. Seal the blotted Zeta-Probe membrane inside a heat-sealable plastic bag. Prepare the following solution for prehybridization:

   50% formamide
   0.12 M Na_2HPO_4, pH 7.2
   0.25 M NaCl
   7% (w/v) SDS
   1 mM EDTA
2. Cut one corner of the plastic bag and pipet the prehybridization solution in, then reseal the bag.
3. Incubate at 43°C for 5 min.

Hybridization

1. Cut one corner of the bag, remove the prehybridization solution, and replace it with the same buffer.
2. Add probe, then seal the open corner (taking care to exclude all air bubbles). Mix the contents of the bag thoroughly. Incubate at 43°C for 4–24 hr with agitation.

   Note: At no stage before washing should the membranes be permitted to dry.

Washes

1. At the completion of hybridization, remove membranes from their hybridization bags and place them in 2x SSC. Rinse briefly, then wash them successively by vigorous agitation at room temperature for 15 min in each of the following solutions:

   2x SSC/0.1% SDS
   0.5x SSC/0.1% SDS
   0.1x SSC/0.1% SDS

Note: For single-copy detection or high stringency, conduct the last wash at 65°C.

2. After washing, the blotted membranes are ready for autoradiography. If no further cycles of hybridization are to be done on the membrane, the membrane can be dried. When reprobing, do not allow the membrane to dry between hybridizations. Expose moist membranes between plastic wrap or enclosed in a sealable plastic bag. Do not allow a wet membrane to come in contact with the film, because wet Zeta-Probe membrane will stick to the film.

4.3 Alternative Protocol

In this section two hybridization protocols using hybridization accelerators are presented. When extreme hybridization sensitivity is needed, these accelerators will help to increase the target signal by acting as volume excluders. Hybridization accelerators will also decrease the hybridization time needed. In some applications, hybridization accelerators can reduce the hybridization time from overnight to 4 hr. It is suggested that you first work with the standard hybridization protocol (Section 4.1) and determine if your experiments require a hybridization accelerator before using the following protocols.

1. Polyethylene glycol (PEG)[15]— follow the instructions for standard hybridization (Section 4.1) or formamide hybridization (Section 4.2) except add 10% (w/v) PEG 8,000 MW into the hybridization solution in step 1.

   Conduct post-hybridization washes as described in Section 4.1 or 4.2, without PEG.

2. Dextran sulfate — follow the instructions for formamide hybridization (Section 4.2) except increase the hybridization temperature to 65°C and substitute the following prehybridization and hybridization solutions in step 1:

   2x SSPE
   1% (w/v) SDS
   0.5% (w/v) BLOTTO
   10% (w/v) dextran sulfate
   0.5 mg/ml nonhomologous carrier DNA

4.4 Oligonucleotide Protocol[6]

Prehybridization

1. Seal the blotted Zeta-Probe membrane inside a heat-sealable plastic bag. Prepare the following solution for prehybridization:

   5x SSC
   20 mM Na_2HPO_4, pH 7.2
   7% SDS
   1x Denhardt's
   100 µg/ml denatured herring sperm DNA

   The carrier DNA must be denatured before adding it to the hybridization solution by heating at 100°C for 5 min, followed by rapid cooling on ice.

2. Cut one corner of the plastic bag and pipet prehybridization solution in, then reseal the bag.

3. Incubate at 50°C for 0.5–24 hr.

Hybridization

1. Immediately before use, fragment and denature the probe and carrier DNA as follows. Dissolve the radiolabeled probe in 0.1 ml of 0.2 M NaOH, add carrier DNA, mix, and centrifuge briefly to consolidate the solution. Pierce a fine hole in the tube cap and place the tube in a heating block at 100°C for 5 min, followed by rapid cooling on ice.

2. Cut one corner of the bag, remove the prehybridization solution, and replace it with the same buffer.
3. Add probe, then seal the open corner (taking care to exclude all air bubbles). Mix the contents of the bag thoroughly. Incubate at 50°C for 4–24 hr.

   Note: At no stage before washes should the membranes be permitted to dry.

Washes

1. Wash the membrane twice at 50 °C for 30 min in the following:

   3x SSC
   10x Denhardt's
   5% SDS 25 mM NaH_2PO_4, pH 7.5
2. Wash the membrane once at 50 °C for 30 min in the following:

   1x SSC
   1% SDS
3. After washing, the blotted membranes are ready for autoradiography. If no further cycles of hybridization are to be done on the membrane, the membrane can be dried. When reprobing, do not allow the membrane to dry between hybridizations. Expose moist membranes between plastic wrap or enclosed in a sealable plastic bag. Do not allow a wet membrane to come in contact with the film, because wet Zeta-Probe membrane will stick to the film.

Section 5
Hybridization Protocol for RNA Probes

Prehybridization

1. Seal the blotted Zeta-Probe membrane inside a heat-sealable plastic bag. Prepare the following solution for prehybridization:

   50% formamide
   1.5x SSPE
   1% SDS
   0.5% BLOTTO
   0.2 mg/ml carrier RNA
   0.5 mg/ml carrier DNA

   The carrier DNA must be denatured before adding it to the hybridization solution by heating at 100°C for 5 min, followed by rapid cooling on ice.
2. Cut one corner of the plastic bag and pipet prehybridization solution in, then reseal the bag.
3. Incubate at 50°C for 0.5–24 hr.

Hybridization

1. Immediately before use, fragment and denature the probe and carrier DNA as follows. Add to the precipitated RNA probe 0.1 ml of yeast RNA (20 mg/ml), 0.5 ml of carrier DNA (10 mg/ml), and 0.6 ml of deionized formamide, mix thoroughly, and heat at 70°C for 5 min.
2. Cut one corner of the bag, remove the prehybridization solution, and replace it with hybridization buffer:

   50% formamide
   1.5x SSPE

1% SDS
0.5% BLOTTO

3. Add probe, then seal the open corner (taking care to exclude all air bubbles). Mix the contents of the bag thoroughly. Incubate at 50°C for 4–24 hr.

   Note: At no stage before washing should the membranes be permitted to dry.

Washes

1. At the completion of hybridization, remove the membranes from their hybridization bags and place them in 2x SSC. Rinse briefly, then wash them successively by vigorous agitation for 15 min at room temperature in the following solutions:

   2x SSC/0.1% SDS
   0.5x SSC/0.1% SDS
   0.1x SSC/0.1% SDS

2. After washing, the blotted membranes are ready for autoradiography. If no further cycles of hybridization are to be done on the membrane, the membrane can be dried. When reprobing, do not allow the membrane to dry between hybridizations. Expose moist membranes between plastic wrap or enclosed in a sealable plastic bag. Do not allow a wet membrane to come in contact with the film, because wet Zeta-Probe membrane will stick to the film.

   Note: To increase the rate of hybridization, include 10% dextran sulfate (final concentration) in the hybridization solution. Prewarm the hybridization solution to 50°C. Denature the probe and carrier as above. Special care must be taken to insure uniform mixing of the denatured probe with the hybridization solution, since the solution is quite viscous at 50°C.

Section 6
Probe Stripping and Rehybridization

If reprobing is desired, do not allow the Zeta-Probe membrane to dry between hybridizations.

The Zeta-Probe membrane should be stripped as soon as possible after autoradiography.

Wash the membrane twice for 20 min each in a large volume of 0.1x SSC/0.5% SDS at 95°C.[2] Check membrane by overnight exposure.

Section 7
Zeta-Probe Membrane Troubleshooting Guide

7.1 Nucleic Acids Problem

Problem	Solution
1. Fragments greater than ~1,000 bp cannot be electrophoretically transferred from polyacrylamide gels after base denaturation, even at increased volts/hours	We have observed that fragments >~1,000 bp can become trapped in polyacrylamide gels if they are base denatured and neutralized after electrophoresis, whereas nondenatured fragments will transfer completely up to at least 2,000 bp. Solve this problem in one of three ways: 1. Omit pretreatment, and transfer ds DNA. Alkaline fix post-blotting. 2. Run gel electrophoresis under denaturing conditions, and omit base denaturation step and neutralization step prior to transfer. 3. Omit base denaturation step, and denature gel instead with 1 M glyoxal, in 25 mM sodium phosphate, pH 6.5, 50% DMSO for 1 hr at 50°C. Then transfer directly.
2. Very large fragments cannot be electrophoretically eluted from agarose gels	1. Solutions 1 and 2 to problem #1 can also be applied to agarose gels. 2. Depurinate prior to transfer by soaking the gel in 0.25 M HCl for 20 min,[6] then soak the gel in transfer buffer for 10–15 min.

Nucleic Acids (Continued)

Problem	Solution
3. High background observed throughout membrane on autoradiograph	The major contributors to background are unincorporated label, small radioactive decay products, and small probe fragments resulting from nick translation or random priming. 1. Use a desalting gel column to remove unincorporated label. Bromophenol Blue is a useful indicator. The peak of unincorporated label overlaps with, and slightly precedes the Bromophenol Blue in a desalting column. 2. Use the probe as soon as possible after preparation, since decay results in fragmentation. 3. Reduce exposure of the probe to DNase I during nick translation to increase average probe length. 4. Use a different heterologous nucleic acid in the prehybridization and hybridization mixtures. Sonicate it thoroughly and denature it before use. 5. For RNA probe, post-hybridization washes: remove SDS by washing 3x in 100 mM sodium phosphate (pH 7.2), then wash with 10 µg/ml RNase A, 0.3 M NaCl, 10 mM Tris-HCl, pH 7.5, 1 mM Na_2EDTA at 37°C for 15 min.

Nucleic Acids (Continued)

Problem	Solution
4. Localized high background observed on autoradiograph	1. Make sure the membrane is free-floating within the plastic bag during hybridization. Membrane/bag contact during hybridization can cause background. Add more hybridization solution. 2. Make sure not to pinch the membrane when sealing the plastic bag prior to hybridization. 3. Be sure no bubbles exist in the hybridization bag.
5. Lane background or extra bands	1. Indicates contaminated template. Make sure the probe is synthesized with the pure template of choice.
6. Low autoradiograph signal	This problem may occur when total genomic DNA is probed for single-copy or low copy number genes. 1. Incorporate 10% dextran sulfate in the hybridization mixture. This polymer effectively reduces the solvent volume, thereby increasing the concentration of the solutes and enhancing hybridization. Refer to Section 4.3. 2. Increase exposure time to increase signal-to-noise ratio. 3. Increase sample load on the gel. 4. If low signal is accompanied by low background, probe concentration can be increased 2- to 4-fold.

Problem	Solution
7. No autoradiograph signal	1. After transfer, stain the gel to check that transfer was complete. If not, increase transfer time and/or voltage of transfer, or see solution to problem #1 above. 2. Be sure probe is denatured by boiling or heating to 65°C for 5 min in 50% formamide prior to hybridization.

Section 8
Appendix

20x TAE	MW	g/L
0.8 M Tris	121.1	96.9
0.4 M base sodium acetate	82.04	32.8
20 mM EDTA	372.2	7.45

pH to 7.4 with glacial acetic acid

5x TBE	MW	g/L
0.5 M boric acid	61.8	30.9
0.5 M Tris base	121.1	60.5
10 mM EDTA	372.2	3.73

20x SSC	MW	g/L
3 M NaCl	58.44	175.0
0.3 M trisodium citrate	294.1	88.2

20x SSPE	MW	g/L
3.6 M NaCl	58.44	210.0
0.2 M $Na_2HPO_4 \cdot 7\ H_2O$	268.07	53.6
20 mM EDTA	372.2	7.44

TE

10 mM Tris-HCl, pH 8.0
1 mM EDTA, pH 8.0

100x Denhardt's Solution	MW	g/100 ml
2% bovine serum albumin		2
2% polyvinylpyrrolidone	360,000	2
2% Ficoll	400,000	2

10% BLOTTO	g/100 ml
Nonfat powdered milk	10
0.2% sodium azide	0.2

Store at 4°C

20% SDS	MW	g/L
20% sodium dodecyl sulfate	288.38	200

Heat to 65°C to get into solution

1 M Na_2HPO_4, pH 7.2	MW	g/L
1 M $Na_2HPO_4 \cdot 7\ H_2O$	268.07	268.07

Add 4 ml 85% H_3PO_4 [1 M in Na^+, see Reference 4]

50% Dextran Sulfate	MW	g/100 ml
50% dextran sulfate	500,000	50
0.2% sodium azide	65.01	0.2

Store at 4°C

50% Formamide	g/100 ml
50% formamide	50

Store at 4°C. Immediately before use, deionize the required volume by stirring gently for 1 hr with 1 g mixed-bed ion exchange resin (AG® 501-X8(D) resin, catalog #142-6425) per 10 ml of formamide. Filter through coarse filter paper.

6 M Glyoxal (Deionized)

Deionize 6 M glyoxal by pouring over a small mixed-bed resin column (AG 501-X8 mixed bed ion exchange resin, catalog #142-6424). Store at −20°C in small aliquots. Once an aliquot has been exposed to air, it cannot be reused.

Section 9
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Section 10
Ordering Information

Catalog Number	Product Description
162-0153	**Zeta-Probe Membranes,** 9 x 12 cm, 15 sheets
162-0154	**Zeta-Probe Membranes,** 10 x 15 cm, 15 sheets
162-0155	**Zeta-Probe Membranes,** 15 x 15 cm, 15 sheets
162-0156	**Zeta-Probe Membranes,** 15 x 20 cm, 15 sheets
162-0157	**Zeta-Probe Membranes,** 20 x 20 cm, 15 sheets
162-0158	**Zeta-Probe Membranes,** 20 x 25 cm, 3 sheets
162-0159	**Zeta-Probe Membranes,** 30 cm x 3.3 m roll
162-0165	**Zeta-Probe Membranes,** 20 cm x 3.3 m roll
165-5000	**Model 785 Vacuum Blotter**
165-5031	**GS Gene Linker UV Chamber,** 120 VAC
165-5052	**PowerPac™ 200 Power Supply,** 100/120 VAC
170-6545	**Bio-Dot® Microfiltration Apparatus**
170-6542	**Bio-Dot SF Microfiltration Apparatus**
162-0133	**Molecular Biology Certified Agarose,** 100 g
162-0134	**Molecular Biology Certified Agarose,** 500 g
161-0733	**10x Tris/Boric Acid/EDTA,** 1L
161-0301	**SDS (Sodium Dodecyl Sulfate),** 100 g
161-0302	**SDS (Sodium Dodecyl Sulfate),** 500 g
732-6000	**Bio-Spin 6 Columns,** 10
732-6004	**Bio-Spin 30 Columns,** 10
142-6425	**AG 501-X8 (D) Resin,** 500 g

Related Proceedings Appendix

The following United States patents related to the application involved in the present appeal: patent No. 5,888,819; patent No. 6,004,744; and patent No. 6,537,748; are the subject of a pending patent infringement lawsuit: Beckman Coulter Inc. and Orchid Cellmark Inc. v. Sequenom, Inc., No. 08 CV 1013 W POR (S.D. Cal. 5 June 2008). So far as the undersigned attorney is aware, no final decision has been issued with respect to the lawsuit.

Interference 103,739 was terminated pursuant to a judgment in favor of the junior party dated 26 March 1998, a copy of which is enclosed.

THIS OPINION WAS NOT WRITTEN FOR PUBLICATION

The opinion in support of the decision being entered today (1) was not written for publication in a law journal and (2) is not binding precedent of the Board.

Paper No. 33

UNITED STATES PATENT AND TRADEMARK OFFICE

BEFORE THE BOARD OF PATENT APPEALS AND INTERFERENCES

PHILIP GOELET, MICHAEL R. KNAPP and STEPHEN ANDERSON

Junior Party,[1]

v.

HANS E. SODERLUND and ANNE-CHRISTINE SYVANEN

Senior Party.[2]

Interference No. 103,739

Before URYNOWICZ, SOFOCLEOUS and DOWNEY, Administrative Patent Judges.

SOFOCLEOUS, Administrative Patent Judge.

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[1] Application 07/664,837, filed March 5, 1991. Assignor to Molecular Tool, Inc., a Corporation of Delaware.

[2] Application 08/162,376, filed December 2, 1993. Accorded Benefit of U.S. Application Nos. 07/656,575, filed February 15, 1991, now abandoned; and 07/482,005, filed February 16, 1990, now abandoned. Assignor to Orion Corporation, Ltd., a Corporation of Finland.

Interference No. 103,739

JUDGMENT

Molecular Tool, Inc., the common assignee of the parties Goelet et al. and Soderlund et al., has filed a request for entry of an adverse judgment with respect to the senior party Soderlund et al. Pursuant to 37 CFR § 1.662(a), judgment as to the subject matter of the count in issue is hereby awarded to Philip Goelet, Michael R. Knapp and Stephen Anderson, the junior party. Accordingly, Hans E. Soderlund and Anne-Christine Syvanen, the senior party, are not entitled to a patent containing claims 102 to 140 corresponding to the count.

STANLEY M. URYNOWICZ, JR. Administrative Patent Judge	
MICHAEL SOFOCLEOUS Administrative Patent Judge	BOARD OF PATENT APPEALS AND INTERFERENCES
MARY F. DOWNEY Administrative Patent Judge	

Interference No. 103,739

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